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RESEARCH ARTICLE

Galacto-oligosaccharides may directly enhance intestinal barrier function through the modulation of goblet cells

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Scope: Here we have tested the hypothesis that prebiotic galacto-oligosaccharides (GOS) may enhance mucosal barrier function through direct modulation of goblet cell function.
Methods and results: Human adenocarcinoma-derived LS174T cells, which exhibit an intestinal goblet cell-like phenotype, were used to examine the non-prebiotic effects of GOS on goblet cell functions. LS174T cells were treated with GOS, and the expression of goblet cell secretory product genes mucin 2 (*MUC2)*, trefoil factor 3 (*TFF3)*, resistin-like molecule beta (*RETNLB*) and the Golgi-sulfotransferase genes, carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 5 (*CHST5)* and galactose-3-O-sulfotransferase 2 (*GAL3ST2*), was determined by real-time quantitative RT-PCR. In addition, the abundance of CHST5, TFF3 and RETNLB was confirmed by Western blot analysis. Following treatment with GOS for 72 h, the expression of *MUC2* was significantly upregulated 2–4-fold, *CHST5* and *RETNLB*, 5–7-fold, and *TFF3* 2–4-fold. Western blot analysis demonstrated increased abundance of RETNLB, TFF3 and CHST5. Addition of the Th2 cytokine IL-13 along with GOS resulted in synergistic induction of *RETNLB* and *CHST5*. IL-8 secretion was not affected by GOS treatment, suggesting that the effects of GOS are not mediated through an inflammatory pathway.
Conclusion: Collectively, the data indicate that GOS may enhance mucosal barrier function through direct stimulation of intestinal goblet cells.

Keywords:
Galacto-oligosaccharides / Golgi-sulfotransferases / LS174T goblet cells / *RETNLB* / RELMβ

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1 Introduction

Oligosaccharides constitute a major component of many natural foods including human milk. Many of these undergo digestion in the small intestine; however, certain oligosaccharides such as galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS) and human milk oligosaccharides (HMO), are resistant to digestion by intestinal enzymes due to their unique chemical structure [1, 2]. Such oligosaccharides are classified as ''prebiotics,'' a term commonly used to describe a nondigestible food ingredient that beneficially

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Abbreviations: **CHST5**, carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 5; **FOS**, fructo-oligosaccharides; **GAL3ST2**, galactose-3-O-sulfotransferase 2; **GOS**, galacto-oligosaccharides; **HMO**, human milk oligosaccharides; **MUC2**, mucin 2; ***RETNLB***, gene name for RELM-β, resistin-like molecule beta; **TFF3**, trefoil factor 3

affects the host by stimulating the growth or activity of a limited number of bacteria in the colon [3, 4].

Several prebiotics, including GOS, are bifidogenic [3, 5], leading to their use in infant formula as a functional replacement for HMOs [6–8]. Moreover, GOS is structurally similar to cell surface glycoconjugates that are used by pathogens for adherence in the gut and, in this way, protect against the colonization and growth of pathogens [9–11]. However, the ability of GOS to enhance mucosal barrier function directly, potentially via its impact on goblet cell function, has not been explored previously. Therefore, in the present study we examined the extent to which GOS regulates the expression of goblet cell specific genes and secretory products. We also examined the combined effects of GOS and the mucogenic cytokine IL-13 on the expression of goblet cell secretory products and Golgi-sulfotransferases.

Goblet cells are columnar epithelial cells that specialize in secretion of high molecular weight glycoproteins called mucins consisting of a protein core rich in threonine, proline or serine residues supplemented with an O-linked oligosaccharide [12]. The thick mucus gel layer formed by mucins maintains the integrity of the gastrointestinal mucosal surface and acts as a medium for protection, lubrication, and transport between luminal contents and the epithelial lining. MUC2 is the major mucin synthesized and secreted by intestinal goblet cells [13, 14]. *MUC2* gene expression and secretion can be modulated by several factors including toxins, microbial products, hormones, cytokines, bile salts, and other growth factors [14–16]. In addition to mucins, RELMβ and TFF3 constitute other goblet cell secretory products with distinct functions. The trefoil factor (TFF) family consists of small peptides (6.5–12 kDa) with three intramolecular disulphide bonds. TFF3, also referred to as intestinal trefoil factor, plays a role in epithelial restitution, mucosal protection, and enhancement of the structural integrity of the mucosal barrier by binding to the vWFC domain of MUC2 and increasing mucus viscosity [17–20]. RELMβ belongs to the family of resistin-like molecules (RELMs), which share a unique cysteine-rich C terminus homologous to that found in the adipocyte hormone resistin. RELMβ is an intestine-specific protein, encoded by the gene *RETNLB* and synthesized and secreted as a homodimer by goblet cells; it is highly induced by enteric bacterial colonization and helminth infection [21, 22].

The LS174T goblet cell line used in the present study synthesizes and secretes mucin, specifically MUC2, under normal growth conditions [16, 23, 24]. As a late step in mucus biosynthesis, mucin sulfation occurs within the *trans*-Golgi apparatus. Galactose-3-*O*-sulfotransferase 2 (*GAL3ST2*) transfers sulfate from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to mucin *O*-glycans at the C-3 position of galactose residues, and carbohydrate (*N*-acetylglucosamine 6-*O*) sulfotransferase 5 (*CHST5*) to the C-6 position of *N*-acetylglucosamine [25, 26]. While *CHST5* is abundant in human small intestine and colon and shows preference for sulfation of mucin O-glycans, *GAL3ST2* has been linked with sulfomucin synthesis and its expression was found to be downregulated in nonmucinous adenocarcinoma [27–29]. LS174T cells express both *CHST5* and *GAL3ST2* [30].

The aim of the present study was to determine the effects of GOS on goblet cell function by examining expression of goblet cell secretory product genes *MUC2*, *TFF3*, *RETNLB*, and the Golgi-sulfotransferase genes, *CHST5* and *GAL3ST2*, in the colon adenocarcinoma LS174T cell line, which exhibits a goblet cell-like phenotype.

2 Materials and methods

2.1 Reagents and cell culture

The human LS174T colorectal cancer cell line was obtained from the American Type Culture Collection (ATCC). LS174T cells were maintained in MEM supplemented with 10% Fetalplex (Gemini Bio-Products, West Sacramento, CA), 1.5 g/L of Na_2CO_3, 10 mL/L penicillin G-streptomycin solution (Gemini Bio-Products) at 37°C in 5% CO_2. GOS (Purimune GO-P90) was obtained from GTC Nutrition (Westchester, IL). Purimune™ is a highly pure and soluble galactooligosaccharide powder derived from milk lactose containing a minimum of 90% GOS. It was dissolved in cell culture grade water to the required concentration. The solution was subsequently filter sterilized and used for cell culture studies. The endotoxin level of GOS solution was measured by LAL assay kit (GenScript USA, Piscataway, NJ) and found to be <0.5 EU/mL (endotoxin units/mL). For immunostimulation experiments, IL-13 (PeproTech, Rocky Hill, NJ) was used at a concentration of 100 ng/mL. For each experiment, LS174T cells were seeded in 24-well plates and grown to around 80% confluence. Prior to treatment, cells were rinsed with 1× Hank's balanced salt solution (Sigma-Aldrich, St. Louis, MO), after which 0.5 mL of fresh medium containing various treatments was added to the cultures. Cells were harvested at the end of a 72 h incubation period with 1 mL of TRIzol (Invitrogen, Grand Island, NY).

2.2 RNA isolation and reverse transcription

Total RNA was isolated from TRIzol-suspended LS174T cells using the RNeasy Plus Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The quality and quantity of RNA isolates were determined by Nanodrop (Thermo Fisher Scientific, Waltham, MA). RNA isolates were reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) and cDNA synthesized was used for performing quantitative PCR.

2.3 Gene expression

For real-time quantitative RT-PCR, specific TaqMAN gene expression assays (Applied Biosystems) for *MUC2*

(Hs00159374_m1), *TFF3* (Hs00173625_m1), *RELMβ* (Hs00395669_m1), *CHST5* (Hs00375495_m1), *GAL3ST2* (Hs00223271_m1), and *GUSB* (Hs99999908_m1) were used with TaqMAN PCR Master Mix (Applied Biosystems). Reactions were run in duplicate in a 384-well plate using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Results were analyzed using SDS 2.3 software and calculated by the delta delta C_T method. All samples were normalized to the expression of the gene for beta glucuronidase, *GUS-β*, and fold induction was calculated over untreated controls.

2.4 Western blot analysis

For Western blot analysis cells were seeded in 6-well plates and grown to 80% confluence. Cells were then treated with GOS for 96 h. At the end of incubation, cells were scraped from culture plates and lysed with 400 μL of 1× Cell Lysis Buffer (Cell Signaling Technology Inc., Danvers, MA) with protease inhibitor cocktail (Sigma Aldrich Co., St. Louis, MO). Immunoblotting was performed as described earlier [31]. The bound proteins were probed with the following primary antibodies: RELMB (rabbit anti-human at a dilution 0.2 μg/mL; Peprotech, Rocky Hill, NJ), CHST5 (mouse monoclonal antibody at a dilution of 3μg/mL; Abnova, Taipei, Taiwan), TFF3 (mouse monoclonal antibody at a dilution of 2 μg/mL; R&D Systems, Minneapolis, MN), β-actin (mouse monoclonal antibody at a dilution of 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Following incubation, blots were washed with PBST and incubated with respective (anti-rabbit and anti-mouse) horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology) at a dilution of 1:2000 for 1 h at room temperature. After thorough washes in PBST, bands were visualized by treating the membranes with Chemiluminescence (Thermo Scientific, USA). Membranes were photographed and the bands were quantified by densitometric analysis using NIH ImageJ software. Densitometry data presented in bar graphs depict relative expression compared to controls in each case.

2.5 Analysis of CHST5 by immunofluorescence

LS174T cells were seeded in 8 well μ-slides (Ibidi) at a density of 6×10^4 cells/well and allowed to grow for 48 h. Cells were then washed twice with 1× HBSS, and MEM media (without phenol red) containing GOS (8 mg/mL) was added to the cells. After 24, 48, and 96 h, cells were washed three times in 1× PBS and fixed with 4% paraformaldehyde for 20 min. Immunofluorescence staining was performed as described earlier [30]. Cells were rinsed once in 1× PBS, incubated for 2 h at room temperature with a rabbit polyclonal antibody to CHST5 (Novus Biologicals, Littleton, CO) diluted 1:100 in 10% blocking solution, and washed three times in 1× PBS for 5 min each. Incubation with FITC anti-rabbit IgM (Invitrogen) diluted 1:400 in 10% blocking solution was performed for 2 h at room temperature and followed by three washes with 1× PBS for 5 min each. Cells were counterstained with 10 μg/mL of DAPI (Invitrogen) for 15 min, rinsed 3× in 1× PBS, and covered with Prolong Gold Antifade Reagent (Invitrogen) for 24 h in the dark at room temperature. Stained cells were stored in the dark at 4°C prior to imaging with a Zeiss LSM 710 confocal microscope using the 63× oil objective and Zen 2011 software (Zeiss).

2.6 IL-8 secretion

Following treatment of LS174T cells with GOS, secretion of IL-8 was measured in conditioned media using a Human CXCL8/IL-8 Quantikine ELISA Kit (R & D Systems, Minneapolis, MN) according to manufacturer's instructions.

2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.0; San Diego, USA). The results are presented as mean ± SEM. The statistical differences between the groups were determined by one way ANOVA and Fisher's protected least significant difference test for more than two groups. The probability value of $p < 0.05$ was considered significant.

3 Results

3.1 Effects of GOS on gene expression of goblet cell secretory products and Golgi-sulfotransferases

Prior to treatment of LS174T cells with 8 mg/mL GOS for 72 h to measure expression of *MUC2*, *TFF3*, *RETNLB*, and Golgi-sulfotransferases *CHST5* and *GAL3ST2*, optimum incubation times with GOS were determined by treating cells with 20 mg/mL of GOS, equivalent to the concentration of oligosaccharides present in human milk, for time periods from 24 to 96 h. In general, gene expression increased with an increase in incubation time up to 72 h Supporting Information Fig. 1). Further, for optimizing dose, LS174T cells were incubated with 1–20 mg/mL dose of GOS for 72 h. Expression of *MUC2*, *TFF3*, *RETNLβ*, and *CHST5* was induced over untreated controls at doses of 8–20 mg (Supporting Information Fig. 2). Hence, 8 mg/mL GOS was used for all subsequent experiments.

As shown in Fig. 1, while *MUC2* was upregulated 2.8-fold, *TFF3* and *RETNLB* showed a robust induction of 7.2–7.5-fold over untreated controls. While the expression of *CHST5* was also significantly upregulated 2.9-fold, *GAL3ST2* expression did not vary (Fig. 1). Hence, the expression of goblet cell secretory product genes *MUC2*, *TFF3*, *RETNLB*, and Golgi-sulfotransferase *CHST5* were differentially modulated, demonstrating that GOS treatment at 8 mg/mL can affect goblet cell function.

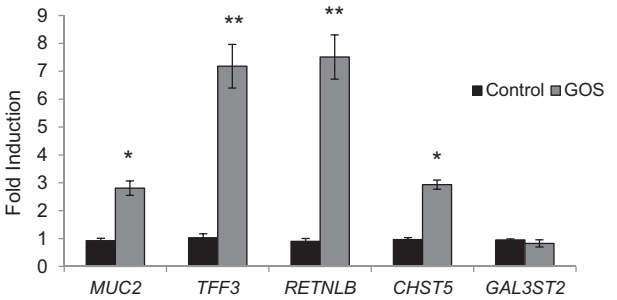

Figure 1. GOS increases expression of goblet cell secretory and Golgi sulfotransferase genes in LS174T cells. LS174T cells were treated with 8 mg/mL GOS for 72 h, and mRNA expression of goblet cell secretory products and Golgi-sulfotransferase genes was calculated over untreated controls. The data represent mean ± SEM ($n = 4$). Asterisks indicate values that are statistically different from control (*$p < 0.05$, **$p < 0.01$).

3.2 GOS enhances RETNLB, TFF3, and CHST5 protein expression

Western blot analysis reveals that CHST5 was induced after 96 h of GOS treatment (Fig. 2A). The level of TFF3 also increased significantly at 96 h (Fig. 2B). Further, when LS174T cells were treated with GOS for 96 h, the abundance of RETNLB also increased significantly (Fig. 2C). The increased abundance of CHST5 in LS174T cells after 96 h of GOS treatment was confirmed by immunofluorescent staining (Fig. 3).

3.3 Effects of GOS and IL-13 on gene expression of goblet cell secretory products and Golgi-sulfotransferases

To explore the effect of prebiotic GOS in combination with the Th2 cytokine IL-13, cells were treated for 72 h with GOS and IL-13 either alone or in combination. As shown in Fig. 4A, GOS alone resulted in a 4-fold increase in *MUC2* expression, while IL-13 did not alter its expression at this time point. However, when both GOS and IL-13 were present, *MUC2* was induced 5.8-fold. *TFF3* was induced 4.7 fold with GOS alone and 1.5-fold with IL-13 alone, while in the presence of GOS + IL-13, *TFF3* was induced 8-fold (Fig. 4B). *RETNLB* expression was induced 5.4-fold with GOS and 2-fold with IL-13 alone, but the presence of both GOS and IL-13 synergistically induced *RETNLB* 13.5-fold (Fig. 4C). Among Golgi-sulfotransferases, expression of *CHST5* was induced by GOS 4-fold and 8-fold by IL-13; however, a synergistic induction of 18.5-fold was observed for *CHST5* expression when GOS and IL-13 were present together (Fig. 4D). As shown in Fig. 4E, *GAL3ST2* expression decreased with GOS, but IL-13 alone or in combination with GOS did not induce any

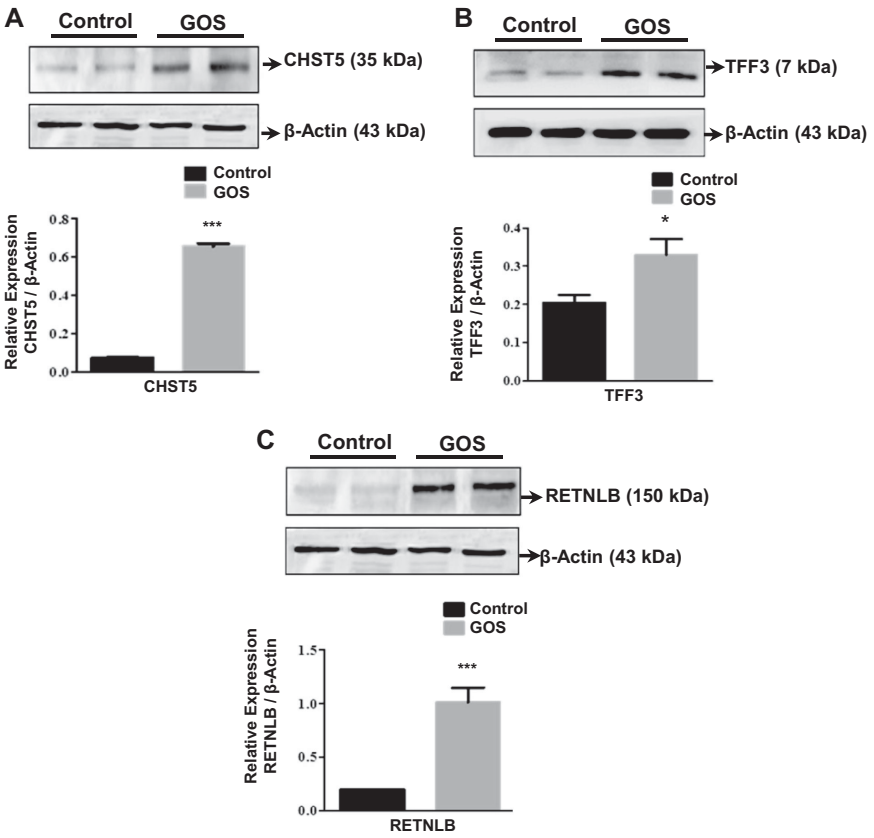

Figure 2. Western blot analysis of CHST5, TFF3, and RETNLB demonstrates increased protein abundance from LS174T cells after 96 h of GOS treatment. Western blot analysis was performed with antibodies against (A) CHST5 (B) TFF3, and (C) RETNLB relative to β-actin. Quantitative data using densitometry are expressed in relative intensity (arbitrary units). The data represent mean ± SEM ($n = 3$). Asterisks indicate values that are statistically different from controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Protein samples analyzed for CHST5, RETNLB and the loading control, β-actin, were size-separated on the same gel and, after transfer, the blots were developed separately for each target.

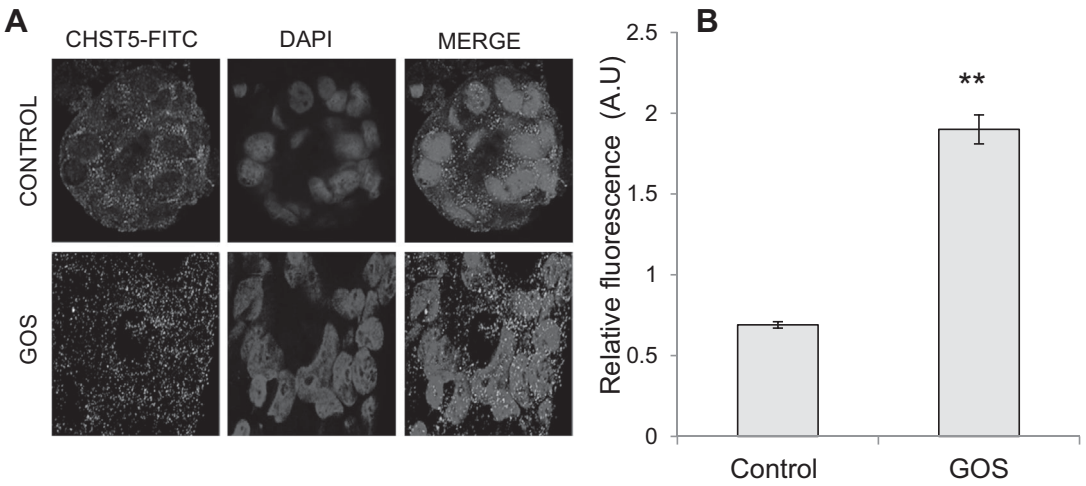

Figure 3. Immunofluorescent staining for CHST5 is increased with 96 h treatment of LS174T cells with GOS. (A) Control and GOS-treated LS174T cells were stained with anti-CHST5 antibody and FITC-conjugated secondary antibody. Cells were counterstained with DAPI. Stained sections were visualized by confocal microscopy (Zeiss LSM 710 at 63× magnification). Color saturation was decreased by 50% to allow DAPI staining to be visible against a black background. Original color image can be viewed in online publication. (B) Quantitative data using Image J software expressed in relative fluorescence arbitrary units (A.U.). The data represent mean ± SEM ($n = 2$). Asterisks indicate values that are statistically different from controls (*$^{**}p < 0.01$*).

significant change over control. Finally, IL-8 secretion, measured from conditioned medium by ELISA, did not vary in response to GOS or IL-13 alone, or with GOS in the presence of IL-13, compared to untreated control cells.

4 Discussion

Oligosaccharides such as GOS have been widely accepted as prebiotic food ingredients, particularly in infant formulas [2]. While prebiotics have been shown to prevent bacterial colonization and invasion by pathogens [2, 11, 32, 33], their role in enhancing mucosal barrier function through direct interactions with intestinal epithelial cells has not yet been explored. The present study examined the effects of GOS on the expression of goblet cell secretory products and Golgi-sulfotransferases. We observed a direct effect of GOS on the colonic adenocarcinoma LS174T cell line, which exhibits a goblet cell-like phenotype. An 8 mg/mL dose of GOS alone resulted in significant induction of *MUC2*, *TFF3*, *RETNLB*, and expression of the Golgi-sulfotransferase *CHST5* gene 72 h post-treatment and increased abundance of the proteins TFF3, RETNLB, and CHST5 96 h post-treatment.

RELMβ has been shown to upregulate *MUC2* transcription and secretion in a TNBS-induced colitis model, thereby potentially strengthening mucosal barrier integrity [34, 35]. In another in vivo study, GOS ingestion by BALB/c mice markedly increased O-linked glycoproteins associated with

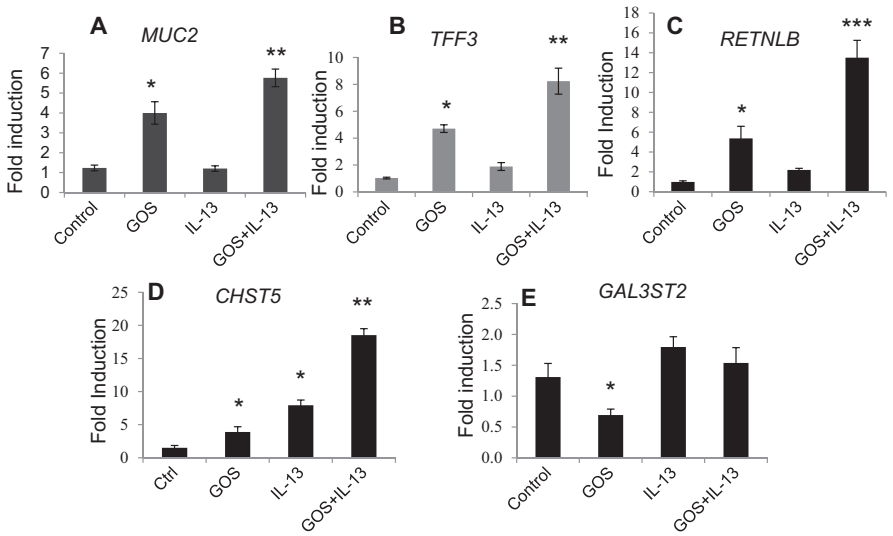

Figure 4. GOS and IL-13 synergistically increase expression of goblet cell secretory genes and one of two Golgi-sulfotransferases from LS174T cells. LS174T cells were treated with GOS in the presence or absence of IL-13 for 72 h, and mRNA expression of goblet cell secretory product and Golgi-sulfotransferase genes was measured. Fold induction of (A) *MUC2* (B) *TFF3* (C) *RETNLB* (D) *CHST5* and (E) *GAL3ST2* in response to GOS was calculated over untreated controls. The data represent mean ± SEM ($n = 3$). Asterisks indicate values that are statistically different from control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

the intestinal mucosa; however, *Muc2* expression was not modified [36]. It is not clear if those effects of GOS in vivo are due to its prebiotic properties or if GOS can directly stimulate expression of goblet cell genes. The present in vitro data demonstrate for the first time that GOS directly modulates the expression of goblet cell secretory products and a Golgi sulfotransferase that contributes to the production of barrier-enhancing sulfomucins. As TFF3 plays a role in epithelial restitution and mucosal protection [18], RELMβ with resolution of inflammation [21, 22], and MUC2 with gastrointestinal barrier function against pathogens [16], it would be appropriate to say that GOS promotes mucosal defense and gastrointestinal integrity via modulation of these genes and their protein products.

Presumably, the direct effects of GOS are mediated by a cell surface receptor(s). Perhaps the best candidate for it is the innate immune pattern recognition receptor dectin-1, a receptor for the glucose polymer β-glucan [37]. Dectin-1 is expressed by various immune cells including dendritic cells, monocytes, macrophages, neutrophils, and a subset of T cells [38]. It contains a single extracellular lectin-like carbohydrate recognition domain and a cytoplasmic tail with an immunoreceptor tyrosine-based activation-like (ITAM-like) motif, which can initiate intracellular signaling upon engagement of β-glucans [37], leading to a range of cellular responses including phagocytosis, respiratory burst, production of arachidonic acid metabolites, and induction of numerous cytokines and chemokines [38]. On the other hand, upon ligand binding dectin-1 activates NFκB [39] and GOS did not upregulate IL-8, which is also a downstream target of NFκB [40]. Further studies are required to determine if dectin-1 or another glycan receptor might mediate the direct effects of GOS on goblet cells.

The Th2 cytokine IL-13 has been associated with an increase in mucus secretion in LS174T cells [30, 41]. We observed induction of *MUC2*, *TFF3*, *RETNLB*, and *CHST5* when LS174T cells were treated with both GOS and IL-13 together. Specifically, *RETNLB* and *CHST5* were induced in a synergistic manner, indicating that GOS and IL-13 may activate distinct receptors on LS174T cells to elicit this response. While this appears to be the first report where the combined effect of GOS and a Th2 cytokine was examined, direct immunological effects of short chain GOS and long chain FOS, provided in vitro evidence for epithelial transfer of prebiotics [42]. Further, in a study analyzing the antiallergic effects of GOS in a mouse model of atopic dermatitis, GOS-fed mice exhibited fewer symptoms of dermatitis, showed higher levels of Th1 cytokines and unchanged levels of Th2 cytokines such as IL-13. GOS inhibited production of IL-1β, IL-6, IL-17, and TNFα but enhanced IL-10 [43]. Earlier we reported induction of *CHST5* upon treatment of LS174T cells with IL-13 as early as 12 h post-treatment [30]. In the present study, IL-13 as well as GOS induced *CHST5* alone and a potentiated response was observed in response to combined treatment with IL-13 and *GOS*.

Prebiotics modulate intestinal microbiota and have a beneficial effect on human health by reducing intestinal inflammation. Caco-2 cells upon treatment with alpha3-sialyllactose or FOS showed reduced levels of IL-12 and expression of IL-8, IL-12p35, and TNFα. These oligosaccharides exhibited anti-inflammatory effects and reduced expression and translocation of NFκB [44]. To determine if GOS modulation of goblet cell function is a function of inflammation, we measured secretion of IL-8, a potent neutrophil chemoattractant. The proinflammatory cytokine TNFα stimulates IL-8 secretion [45], while Th2 cytokines IL-4 and IL-13 significantly reduce IL-8 secretion by human intestinal epithelial cells [46]. Exposure to GOS and inulin prebiotic mixture at perinatal and postweaning periods differentially modulated immune responses in mice toward a mechanism to prevent various immune pathologies such as allergies, autoimmune diseases and inflammatory bowel disease [47]. Besides, intestinal epithelial cell lines exposed to various prebiotics including GOS modulated the production of proinflammatory cytokines and MCP 1 (monocyte chemoattractant protein 1) by activating TLR4 and NFκB, thereby confirming that prebiotics are TLR4 ligands in intestinal epithelial cells and may exhibit their in vivo effect by this relevant mechanism [48]. In the present study, IL-8 levels remained unchanged when LS174T cells were treated with GOS alone or in response to IL-13 and GOS together. This outcome indicates that GOS modulation of goblet cell function is not likely a reflection of intracellular signaling through an NFκB-mediated inflammatory cascade [40]. As such, these data establish for the first time direct interaction of GOS with goblet cells as a previously unknown mechanism through which GOS might promote gastrointestinal defense. Though GOS did not appear to act through an inflammatory mechanism, the results provide insights for follow-up studies on more specific aspects. We believe that GOS by enhancing the expression of goblet-cell secretory products makes the intestine more adept for defense against pathogens. Collectively, the present findings identify a novel mechanism by which a ''functional food'' of physiological importance may enhance intestinal barrier function [49]. Follow-up studies are needed to identify the receptors that mediate these effects and to determine the extent to which the effects are also manifested in vivo.

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