Human Milk Oligosaccharides and Synthetic Galactosyloligosaccharides Contain 3'-, 4-, and 6'-Galactosyllactose and Attenuate Inflammation in Human T84, NCM-460, and H4 Cells and Intestinal Tissue Ex Vivo\(^1,2\)

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Abstract

**Background:** The immature intestinal mucosa responds excessively to inflammatory insult, but human milk protects infants from intestinal inflammation. The ability of galactosyllactoses (galactosyloligosaccharides (GOS)), newly found in human milk oligosaccharides (HMOS), to suppress inflammation was not known.

**Objective:** The objective was to test whether GOS can directly attenuate inflammation and to explore the components of immune signaling modulated by GOS.

**Methods:** Galactosyllactose composition was measured in sequential human milk samples from days 1 through 21 of lactation and in random colostrum samples from 38 mothers. Immature [human normal fetal intestinal epithelial cell (H4)] and mature [human metastatic colonic epithelial cell (T84) and human normal colon mucosal epithelial cell (NCM-460)] enterocyte cell lines were treated with the pro-inflammatory molecules tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β) or infected with *Salmonella* or *Listeria*. The inflammatory response was measured as induction of IL-8, monocyte chemoattractant protein 1 (MCP-1), or macrophage inflammatory protein-3α (MIP-3α) protein by ELISA and mRNA by quantitative reverse transcriptase-polymerase chain reaction. The ability of HMOS or synthetic GOS to attenuate this inflammation was tested in vitro and in immature human intestinal tissue ex vivo.

**Results:** The 3 galactosyllactoses (3'GL, 4-GL, and 6'-GL) expressed in colostrum rapidly declined over early lactation (\(P < 0.05\)). In H4 cells, HMOS attenuated TNF-α- and IL-1β–induced expression of IL-8, MIP-3α, and MCP-1 by 48–51% and pathogen-induced IL-8 and MCP-1 to 26–30% of positive controls (\(P < 0.001\)). GOS reduced TNF-α– and IL-1β–induced inflammatory responses to 25–26% and pathogen-induced IL-8 and MCP-1 to 36–39% of positive controls (\(P < 0.001\)). GOS and HMOS mitigated nuclear translocation of nuclear transcription factor κB (NF-κB) p65. HMOS quenched the inflammatory response to *Salmonella* infection by immature human intestinal tissue ex vivo to 26% and by GOS to 50% of infected controls (\(P < 0.01\)).

**Conclusion:** Galactosyllactose attenuated NF-κB inflammatory signaling in human intestinal epithelial cells and in human immature intestine. Thus, galactosyllactoses are strong physiologic anti-inflammatory agents in human colostrum and early milk, contributing to innate immune modulation. The potential clinical utility of galactosyllactose warrants investigation.

**Keywords:** Human milk oligosaccharides, enteric infection, human intestinal epithelium, mucosal immune response, colostrum, galactosyllactose

Introduction

Breastfed infants have lower risk of infection and inflammatory diseases than those fed artificially. In addition to nutrients, human milk molecules modulate immune maturation of the developing intestinal mucosa (1–3). Inflammatory stimulation by newly colonizing bacteria in the newborn gut lacks adequate feedback inhibition, often causing mucosal damage (3, 4). However, many bioactive components of human milk can attenuate intestinal inflammation (4–8). Human milk contains pathogen-specific secretory IgA, lactoferrin, and TGs that release FFAs in the infant stomach, all of which inhibit infection (3–6). Additional human milk components quench excessive inflammation (7, 8). Synthetic GOSs have potential for use as anti-inflammatory agents in the gut, as well as in other diseases that are relieved by suppression of NF-κB signaling (9, 10). In addition, GOSs have potential for dental benefits, as has been shown for solutions of GOSs in water and an oral gel (11–13). In this study, we measure GOS composition over early lactation and assess whether HMOS or GOSs can directly attenuate inflammation in immature and mature human intestinal epithelial cells by examining the components of immune signaling modulated by GOSs.
inflammatory protein-3, innate and adaptive immune responses (2, 3, 15–17). Elevated release cytokines and chemokines of the type that mediate both specific galactosyllactose that inhibits specific inflammatory human gut mucosa ex vivo (9). The most pertinent example is a HMOS can modulate multiple signaling pathways in immature born intestinal mucosa (5, 13, 14). Prior studies indicate that attenuate the excessive inflammatory predilection of the new-born intestinal mucosa (5, 13, 14). Prior studies indicate that inflammation in vitro (3–6), but their mechanisms of action remain indeterminate.

Among the plethora of heterogeneous complex glycans in human milk are oligosaccharides, which have anti-infectious (1, 4) and prebiotic activities (9–12). Prebiotics are indigestible dietary glycans that facilitate colonization by health-promoting beneficial microbes while being fermented into small organic acids that inhibit enteropathogens, thereby indirectly reducing inflammation (3, 13, 14). In addition to this indirect attenuation of inflammation, the human milk oligosaccharide fraction [human milk oligosaccharide (HMOS)]6 may act to directly attenuate the excessive inflammatory predilection of the new-born intestinal mucosa (5, 13, 14). Prior studies indicate that HMOS can modulate multiple signaling pathways in immature human gut mucosa ex vivo (9). The most pertinent example is a specific galactosylactose that inhibits specific inflammatory signaling elicited by activation of Toll-Like Receptor-3 in cultured human enterocytes (9). This suggested the possibility of multiple oligosaccharides of human milk having distinct heterogeneous functions, including modulation of various inflammatory signaling pathways.

When enterocytes encounter pro-inflammatory stimuli, they release cytokines and chemokines of the type that mediate both innate and adaptive immune responses (2, 3, 15–17). Elevated levels of neutrophil chemotactants, such as IL-8, macrophage inflammatory protein-3α (MIP-3α), and monocyte chemottractant protein 1 (MCP-1), are released by epithelial cells and macrophages to recruit activated innate immune cells to the site of injury (18–22). Thus, levels of IL-8, MIP-3α, and MCP-1 induction strongly reflect the degree of pro-inflammatory response after a challenge; suppression of this response by an agent such as HMOS would indicate the ability of the agent to directly quench inflammation.

Of the hundreds of oligosaccharides in human milk, most have no practical alternate source. Therefore, definition of specific functions for HMOS has been limited by availability of adequate quantities of pure oligosaccharides (5, 8, 9). For human milk galactosyllactoses, an alternative practical source is galactosyloligosaccharides (GOS) synthesized from lactose (14). This mixture of GOS contains disaccharides, trisaccharides, tetrasaccharides, and pentasaccharides (23, 24), but we found that their principal components are trisaccharides, specifically the 3 galactosyllactose species identical to those found in colostrum. This commercial GOS (25–28) was used to test the hypothesis that galactosyllactoses of HMOS could directly attenuate inflammatory signaling pathways in the immature gut mucosa; inflammatory signaling was induced by specific pro-inflammatory molecules and by enteric infection. Investigating specific oligosaccharides for potential functions in the intestinal mucosa was most facile in simple reductionist models such as human epithelial cell culture after testing for probable biological relevance in more complex complete tissues like human gut ex vivo models. The inflammatory processes induced by insult in immature neonatal gut are mediated by specific signaling pathways. Physiologic concentrations of HMOS and equal amounts of GOS were investigated for their ability to directly attenuate specific signals that initiate inflammatory cascades of the intestinal mucosa.

6 Abbreviations used: GL, galactosyllactose; GOS, galactosyloligosaccharides; HMOS, human milk oligosaccharides; MCP-1, monocyte chemotactic protein 1; MIP-3α, macrophage inflammatory protein-3α; H4, human normal fetal intestinal epithelial cell; NCM460, human normal colon mucosal epithelial cell; T84, human metastatic colon epithelial cell.

Human milk galactosyllactose and gut inflammation

Methods

Materials. Recombinant TNF-α, streptavidin-HRP, and human CCL20, MIP-3α ELISA development kits were from R&D Systems (Minneapolis, Minnesota). Anti-human IL-8 and mouse anti-human IL-8 antibodies were from Endogen (Woburn, Massachusetts). Trizol and SuperScript III Platinum SYBR Green One-Step qRT-PCR kits; DMEM/F12; DMEM; and CMRL 1066 media, glutamine, HEPES buffer, and penicillin-streptomycin were cell culture grade from Invitrogen (Carlsbad, California). Other reagents included M3D (Incell Corporation, San Antonio, Texas); cellasso, O-phenylenediamine tablets, β-trycin acetate, tricine, gentamicin, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri); FBS (Atlanta Biologicals, Lawrenceville, Georgia); rabbit anti-human NF-κb (p65) polyclonal antibody (Calbiochem, Gibbstown, New Jersey); and Cy 3-conjugated F(ab’)2 fragment goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, Pennsylvania).

Human milk oligosaccharides. HMOS were isolated from 2 L human milk pooled from 40 donors (29, 30) from all stages of lactation, including 7 colostrum samples, 6 from weeks 3–9, 9 from weeks 10–15, 6 from weeks 21–30, 7 from weeks 33–39, and 5 from weeks 42–52. The use of discarded human milk samples for research was granted exempt status by the Institutional Review Board of Boston College (14.024.01e). Fat was removed from the pooled milk by centrifugation, and the bulk of the proteins precipitated by making the skim milk 67% with ethanol at 4°C with stirring for 12 h. After drying, the material was dissolved in 200 mL of water and applied to a charcoal-Celite (1:1) column, the lactose was eluted with 5% ethanol, and the crude oligosaccharides were eluted with 65% ethanol. The ethanol was removed by rotary evaporation, and the oligosaccharides were dried by lyophilization.

Analysis of galactosyllactose by tandem LC-MS. A complete set of sequential early milk samples were obtained from 3 mothers for the first 21 d of lactation, and the galactosyllactose content was quantified. The data from the 3 mothers were combined with analyses of colostrum samples of convenience from 38 mothers to determine the overall range of galactosyllactoses in colostrum samples from the population in the Greater Boston area. The use of discarded human milk samples for research was granted exempt status by the Institutional Review Board of Boston College (14.024.01e). Each 1-mL sample of human milk was centrifuged at 15,000 × g for 15 min at 20°C. A 100-μL aliquot of the skimmed milk was combined with 200 μL methanol, mixed, and put on ice for 30 min. After centrifugation at 15,000 × g for 15 min at 20°C, a 100-μL aliquot of the supernatant was combined with 100 μL of 0.05 mol/L NaBH4, mixed well, and placed on an orbital mixer for 30 min at 20°C. The reduction was stopped with the addition of 100 μL 0.5 mol/L acetic acid and placed on an orbital mixer for 30 min at 20°C. Two milligrams of the hydrogen form of freshly prepared dry Dowex 50WX8 cation exchange resin was added, mixed for 5 min, centrifuged at 15,000 × g at 20°C for 3 min, and taken to dryness. Dry methanol (500 μL) was added followed by evaporation 4 times to remove borate as methyl borate. The dry reduced oligosaccharides were dissolved in 500 μL H2O, mixed, and analyzed by LC-MS. The stationary phase was 3 μm graphitized carbon 100 × 2.1 mm, and the mobile phase was an acetonitrile gradient run at 0.2 mL/min. Solvent A was 0.05% Formic Acid in H2O with NH4OH added to pH 8.5. Solvent B was acetonitrile. The gradient was 10% A for 4 min, 10–17% A for 1.5 min, 17% A for 7.5 min, 17–90% A for 0.5 min, 90% A for 6 min, 90–10% A for 0.5 min, and 10% A for 8 min. The analytes eluted in the order 4-GL, 6’-GL, and 3’-GL from ∼3.5, 7, and 7.5 min, respectively, and were detected in negative mode by specific ion monitoring at m/z = 505 and the chloride adduct m/z = 541 in an Agilent triple-quad mass spectrometer (31).

GOS. GOS from Clasado (Milton Keynes, United Kingdom) had been produced by transgalactosylation of exogenous lactose by the galacto- syltransferases in Bifidobacterium bifidum NCIMB 41171 (a common bacterium in healthy human gut microbiota) (14). Based on analyses presented in results, a 5 mg GOS/mL concentration was considered representative of high physiologic levels in human colostrum.
**Bacterial pathogen culture.** Wild-type Salmonella enterica serovar Typhimurium strain SL1344 (Bobby Cherayil, Massachusetts General Hospital, Boston, Massachusetts) and Listeria monocytogenes (ATCC 19115) were cultured in Luria-Bertani and Brain Heart Infusion broths (Difco), respectively, at 37°C with aeration and prepared for infection (32, 33).

**Human intestinal epithelial cell lines.** Human metastatic colonic epithelial cell (T84) and human normal colon mucosal epithelial cell (NCM-460) cell lines are models of adult human intestinal epithelium; human normal fetal intestinal epithelial cell (H4) cells are models for immature human epithelium, since they are derived from nonmalignant fetal intestinal tissue and exhibit extreme sensitivity to inflammatory stimuli seen in intact immature gut (34). T84 cells were cultured in DMEM/F12; NCM-460 cell medium was M3D, and H4 cells were cultured in DMEM with appropriate supplements (15, 17).

**Activation of NF-κB by luciferase reporter assay.** H4 and NCM-460 cells were transfected with the dual luciferase assay reporter vector pNF-κB-luc and the Renilla luciferase reporter vector pRL-TK, according to the manufacturer’s protocol. Activation of NF-κB by TNF-α was measured by using NF-κB-luciferase reporter expression (35).

**Human intestinal organ culture.** Human small intestine (20–22 wk gestation) was obtained from progestin saline-induced aborted fetuses; maternal informed consent was obtained according to the approved protocol of the Committee for the Protection of Human Subjects from Research Risks of Partners HealthCare (Partners 1999-P003833). Tissues were cultured as organ cultures in CMRL 1066 medium with appropriate supplements (15–17).

**Infection.** Pathogen was cultured and resuspended in antibiotic-free DMEM at a density of ~2 × 10^8 bacteria/mL. Then, 50-μl aliquots of the suspension (~10^6 bacteria) were added to the wells containing the intestinal epithelial cells. The multiplicity of infection was between 5:1 and 10:1 (32, 33). For ex vivo intestinal organ cultures, 1 mL medium per 1 cubic millimeter of tissue was inoculated with 50-μl aliquots containing ~10^7 bacteria. After incubation, intestinal tissues were lysed, serially diluted, cultured on agar plates, and incubated at 37°C for 24 h and bacteria were counted.

**Experimental design**

**HMOS and GOS attenuate chemokine induction.** Intestinal epithelial cells were grown to 70% confluence in 24-well plates from an initial inoculum of 1.5 × 10^5 cells. These cells at 70% confluence are more sensitive to inflammatory stimuli than fully confluent cells. Preliminary dose-response studies for GOS and HMOS had been carried out in T84 cells stimulated with TNF-α and H4 cells stimulated with IL-1β to determine the range of effective doses of HMOS and GOS (not shown), and 5 g HMOS/L, the concentration in human milk, was well within the tested active range of 1–10 g/L and selected for further studies. Quadruplicate wells were used for each variable with induction of inflammation by stimuli [TNF-α (10 μg/L), IL-1β (1 μg/L), Salmonella, or Listeria]: 1) basal negative control, 2) inflammatory stimulus (positive control); 3) HMOS (5 g/L), 4) inflammatory stimulus with HMOS (5 g/L), 5) GOS (5 g/L), 6) inflammatory stimulus with GOS (5 g/L), 7) cellobiose (5 g/L), and 8) inflammatory stimulus with cellobiose. In preliminary experiments, levels of cytokines and chemokines induced by inflammatory stimuli were maximum 16 to 18 h after exposure. After 16–18 h supernatants were collected and stored at ~20°C until IL-8, MCP-1, and MIP-3α were measured.

**Chemokine quantification.** Secreted IL-8, MCP-1, and MIP-3α were measured by ELISA. Chemokine levels were normalized to total cellular protein; and these values were normalized to positive controls to facilitate comparison among experiments with variable expression by positive controls.

**Level of chemokine mRNA measurement.** Total RNA was extracted by Trizol-chloroform, and qRT-PCR was performed to determine the mRNA levels of IL-8, MCP-1, and MIP-3α (9, 16, 17, 36).

**Effect of GOS on NF-κB translocation.** T84 and NCM-460 cells were grown to 70% confluence on coverslips in 6-well plates at an initial concentration of 2.5 × 10^5 cells/mL. Cells were treated in duplicate for 10 and 30 min as follows: 1) negative control, 2) TNF-α (10 μg/L) positive control, 3) GOS, 4) TNF-α with GOS, 5) HMOS, and 6) TNF-α with HMOS. At the end of treatment, cells were fixed in 4% paraformaldehyde, permeabilized with 1% methanol, blocked with 10% goat serum in 0.2% BSA in TBS, and probed with rabbit anti-human NF-κB polyclonal antibody. After 3 TBS washes, cells were incubated with Cy3-conjugated goat anti-rabbit antibody. The coverslips were washed 3 times and mounted on glass microscope slides.

**Effect of HMOS and GOS on NF-κB activation.** H4 and NCM-460 cells were transfected using FuGENE HD (Roche) transiently transfected with indicated plasmids, together with 0.2 μg/well of the reporter plasmid pNF-κB-Luc, and luciferase activity was measured with a dual-luciferase reporter assay system, as described previously (35). The assay was performed in triplicate for each sample with a minimum of 3 independent experiments for each comparison.

**Statistical analysis.** The decreases in concentration of galactosyllactoses in human milk as a function of time were analyzed by generating best-fit regression curves, from which R^2 and P were calculated, all in Microsoft Excel for MAC 14.5.4 (2011). Cytokine secretion from cultured cells, measured per milligram of total cell protein to correct for cell number, was normalized to positive controls and presented as means ± SEMs. Gene expression data obtained by qRT-PCR were expressed as fold increase in specific chemokine mRNA expression over that of GAPDH mRNA expression and presented as mean and SEM. Effects of HMOS and GOS treatment were analyzed by 2-factor ANOVA (Inflammatory stimulus times oligosaccharide treatment) followed by Bonferroni post hoc testing using SPSS statistics version 23. Any data sets that failed Levine’s median test for unequal variance were subjected to logarithmic transformation and restated by Levine’s median test before analysis by ANOVA. Results are expressed as means ± SEMs. P ≤0.05 was considered significant.

**Results**

**Galactosyllactose of early human milk.** Longitudinal milk samples from 3 mothers for the first 21 d of lactation all contained the 3 structural isomers of galactosyllactose, but at different absolute levels among the mothers. Each species declined logarithmically over time (Figure 1). These data were combined with those from 38 mothers to determine the cross-sectional range of galactosyllactoses in colostrum from donors in the Greater Boston area. The highest concentrations were of 6′-galactosyllactose (3–700 μg/mL; median 81, n = 41), whereas the 3′-galactosyllactose (0.5–39 μg/mL; median 4.6, n = 41) and 4-galactosyllactose (0.06–8 μg/mL; median 3.4, n = 41) were present at 2 or 2 orders of magnitude lower concentrations.

**Galactosyllactose content of GOS.** LC-MS revealed >25 peaks from GOS powder, with 69% of their peak area being tri saccharides; of the tri saccharides, 48% were identical to the 3 galactosyllactoses of human colostrum. 3′-GL was 14%, 6′-GL was 12%, and 4-GL was 8% of the total area percentage of all GOS peaks. At least 10 additional minor species comprise the remaining tri saccharides. The total concentration of the 3 galactosyllactoses in human colostrum varies greatly, from ~3.5 to 750 μg/mL in colostrum. These same 3 galactosyllactoses comprised 1.7 g/L when GOS was used as a 5-mg/mL solution. Therefore, a 5-mg GOS/mL solution approximates the high physiologic range of the earliest human colostrum and was deemed an appropriate concentration for exploring possible biological activities.
Enterocyte inflammatory signaling. The ability of HMOS and GOS to attenuate inflammatory induction was tested in T84 and NCM-460 cells, models of enterocytes of mature gut. TNF-α-induced release of pro-inflammatory chemokines and increased their corresponding RNA levels (P < 0.001) (Figure 2). Two-factor ANOVA indicated a significant interaction between TNF-α and oligosaccharides. HMOS and GOS interacted with TNF-α for the induction of IL-8 in T84 cells [F (2, 30) = 17.393, P < 0.0005] and in NCM-460 cells [F (2, 30) = 52.183, P < 0.0005] (Figure 2A, Bi); 2) MIP-3α in T84 cells [F (2, 30) = 162.659, P < 0.0005] and in NCM-460 cells [F (2, 30) = 279.887, P < 0.0005] (Figure 2A, Bii); 3) IL-8 mRNA levels in T84 cells [F (2, 30) = 7.221, P = 0.004] and in NCM-460 cells [F (2, 30) = 13.405, P < 0.0005] (Figure 2C, Di); and 4) MIP-3α mRNA levels in T84 cells [F (2, 30) = 25.525, P < 0.0005] and in NCM-460 cells [F (2, 30) = 44.335, P < 0.0005] (Figure 2C, Dii). Simple main effects analyses (Bonferroni post hoc tests) indicated that induction by TNF-α in NCM-460 cells was attenuated by GOS (IL-8 and MIP-3α reduced induction to 70% of TNF-α controls) and HMOS (IL-8 and MIP-3α reduced induction to 80% and 35% of TNF-α controls, respectively, P < 0.001) (Figure 2B) and their corresponding mRNA levels (Figure 2D). In T84 cells, HMOS reduced TNF-α induction of MIP-3α to 70%, P < 0.03, and GOS reduced induction of IL-8 and MIP-3α to 70% and 50% of TNF-α controls, respectively, P < 0.01 (Figure 2A) and their corresponding mRNA levels (Figure 2C). Cellobiose, the glycan control, did not significantly alter basal or induced levels of pro-inflammatory mediators (not shown). Basal levels of IL-8 and MIP-3α protein or mRNA were not affected by oligosaccharides (Figure 2). The percentage reduction was similar for both protein and mRNA for each pro-inflammatory mediator and all comparisons.

This anti-inflammatory effect was in response to the mixture of molecules that comprise GOS. After incubation with T84 cells, medium was analyzed for the disappearance of specific GOS galactosyllactoses. High-performance anion exchange chromatography/pulsed amperometric detection revealed significant depletion of only the GOS trisaccharide peak from 97 ± 9 to 29 ± 2 absorption units squared (P < 0.001), indicating that GOS activity could be attributed to galactosyllactoses per se (not shown).

Activation and nuclear translocation of NF-κB. Pro-inflammatory signaling by TNF-α, which is mediated by NF-κB, can be measured through an NF-κB-luciferase reporter assay (45). H4 and NCM-460 cells were stimulated with TNF-α with and without HMOS, GOS, and cellobiose, and induction of luciferase protein and mRNA for each pro-inflammatory mediator and their corresponding RNA levels (labeled bars without a common letter differ, P < 0.05) are shown: labeled bars without a common letter differ, P < 0.05. Con, control; GOS, galactosyloligosaccharides; HMOS, human milk oligosaccharides; MIP-3α, macrophage inflammatory protein-3α; NCM-460, human normal colon mucosal epithelial cell; T84, human metastatic colonic epithelial cell.

FIGURE 2 HMOS and GOS mitigated TNF-α–induced IL-8 and MIP-3α protein expression in human T84 (A) and NCM-460 (B) cells, and IL-8 and MIP-3α mRNA levels in T84 (C) and NCM-460 (D) cells. Chemokine secretion, expressed per total cell protein to correct for cell number, was normalized to TNF-α positive controls, and mRNA levels are fold of negative controls (Con). All data are means ± SEMs of 5 independent experiments. Two-factor ANOVA indicated a significant interaction between TNF-α and oligosaccharides (HMOS and GOS) for induction in T84 cells: IL-8 [F (2, 30) = 17.393, P < 0.0005] and MIP-3α [F (2, 30) = 162.659, P < 0.0005] (A); in NCM-460 cells: IL-8 [F (2, 30) = 52.183, P < 0.0005] and MIP-3α [F (2, 30) = 279.887, P < 0.0005] (B); in T84 cells: IL-8 mRNA levels [F (2, 30) = 7.221, P = 0.004] and MIP-3α mRNA levels [F (2, 30) = 25.525, P < 0.0005] (C); in NCM-460 cells: IL-8 mRNA levels [F (2, 30) = 13.405, P < 0.0005] and MIP-3α mRNA levels [F (2, 30) = 44.335, P < 0.0005] (D). Simple main effects analyses (Bonferroni post hoc tests) are shown: labeled bars without a common letter differ, P < 0.05. Con, control; GOS, galactosyloligosaccharides; HMOS, human milk oligosaccharides; MIP-3α, macrophage inflammatory protein-3α; NCM-460, human normal colon mucosal epithelial cell; T84, human metastatic colonic epithelial cell.

FIGURE 1 Galactosyllactoses in early human milk, of which 6'-GL is principal, varied among individual mothers, and all declined logarithmically early in lactation in these representative 3 mothers. Best-fit regression curves, R², describe the decline in concentrations over 21 d, and P indicates the fit between the theoretical curve and the data. 3'-GL, 3'-galactosyllactose; 4-GL, 4-galactosyllactose; 6'-GL, 6'-galactosyllactose.
activity was measured in triplicate. Two-factor ANOVA indicated a significant interaction between TNF-α and oligosaccharides on NF-κB activation in H4 cells \(F(2, 30) = 19.830, P < 0.0005\) (Figure 3A), and in NCM-460 cells \(F(2, 30) = 22.141, P < 0.0005\) (Figure 3B). Simple main effects analyses (Bonferroni post hoc tests) indicated that HMOS attenuated induction to 55% and 38% of TNF-α positive controls in H4 and NCM-460, respectively (\(P < 0.001\)). GOS attenuated induction to 44% and 45% of TNF-α positive controls in H4 (Figure 3A) and NCM-460 (Figure 3B), respectively (\(P < 0.001\)). Cellbiose did not significantly reduce TNF-α induction (\(P > 0.8\)). These data indicate that both HMOS and GOS attenuate activation of NF-κB signaling pathways, thereby preventing release of pro-inflammatory molecules by TNF-α.

Phosphorylation and translocation of NF-κB from the cytoplasm to the nucleus is a central feature of inflammatory signaling. An antibody to the NF-κB p65 subunit allowed its detection by immunofluorescence. Thirty minutes after TNF-α treatment, NF-κB appeared in the nuclei of T84 cells (Figure 3Cii), but the presence of HMOS or GOS caused most of the cellular NF-κB to remain in the cytoplasm (Figure 3Ciii, Civ). Inhibition of translocation persisted for the 1-h duration of the experiment, identical to results in NCM-460 cells (not shown). Inhibition of NF-κB activation, a common inflammatory signal intermediate, suggested that HMOS and GOS might also inhibit inflammation induced by enteric pathogens.

Pathogen-induced chemokine induction in T84 cells. Neither HMOS nor GOS or cellbiose affected viability or infective capacity of *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes*. Infection of T84 cells by enteric pathogens elicits robust induction of IL-8 and MIP-3α, and elevation of their respective mRNA levels (\(P < 0.001\)) (Figure 4). Two-factor ANOVA indicated a significant interaction between oligosaccharides and *Salmonella* infection for the induction of 1) IL-8 \(F(3, 40) = 303.813, P < 0.0005\); 2) MIP-3α \(F(3, 40) = 546.834, P < 0.0005\) (Figure 4A); 3) IL-8 mRNA levels \(F(3, 40) = 127.854, P < 0.0005\); 4) MIP-3α mRNA levels \(F(3, 40) = 10.318, P < 0.0005\) (Figure 4C). Oligosaccharides interact with *Listeria* infection for the induction of 1) IL-8 \(F(3, 40) = 140.221, P < 0.0005\); 2) MIP-3α \(F(3, 40) = 134.963, P < 0.0005\) (Figure 4B); 3) IL-8 mRNA levels \(F(3, 40) = 54.582, P < 0.0005\); 4) MIP-3α mRNA levels \(F(3, 40) = 64.740, P < 0.0005\) (Figure 4D). Simple main effects analyses (Bonferroni post hoc tests) indicated that HMOS attenuated *Salmonella*-induced secretion of IL-8 and MIP-3α to 24% and 26% of infected positive controls, respectively (Figure 4A), and IL-8 mRNA and MIP-3α mRNA levels to 33% and 38% of infected controls, \(P < 0.01\) (Figure 4C). HMOS reduced *Listeria*-induced IL-8 and MIP-3α to 52% and 51% of infected controls, respectively, \(P < 0.01\) (Figure 4B), and IL-8 mRNA and MIP-3α mRNA levels to 36% and 42% of positive controls (Figure 4D). GOS attenuated *Salmonella*-induced IL-8 and MIP-3α to 50% and 47% of infected positive controls, respectively (Figure 4A), and IL-8 mRNA and MIP-3α mRNA levels to 34% and 41% of positive controls, \(P < 0.01\) (Figure 4C). GOS also reduced *Listeria*-induced IL-8 and MIP-3α to 47% and 50% of infected controls, respectively, \(P < 0.01\) (Figure 4B), and IL-8 mRNA and MIP-3α mRNA levels to 49% and 33% of positive controls, \(P < 0.01\) (Figure 4D). The negative control, cellbiose, had no significant effects on the inflammatory response to infection, \(P > 0.84\). Whether HMOS and GOS attenuation of inflammatory signaling also occurs in hyper-responsive immature enterocytes was tested.

**Inflammatory response to TNF-α, IL-1β, and *Salmonella in H4 cells*. The H4 cell line, a model of immature human intestinal epithelial cells, is exquisitely sensitive to pro-inflammatory stimuli (Figure 5). Two-factor ANOVA indicated a significant interaction between the sequelae of these tissue insults and the oligosaccharides. HMOS and GOS interacted with TNF-α for the induction of 1) IL-8 \(F(2, 24) = 391.177, P < 0.0005\); 2) MIP-3α \(F(2, 24) = 519.048, P < 0.0005\); 3) MCP-1 \(F(2, 24) = 503.941, P < 0.0005\) (Figure 5A); 4) IL-8 mRNA levels \(F(2, 24) = 60.646, P < 0.0005\); 5) MIP-3α mRNA levels \(F(2, 24) = 16.947, P < 0.0005\); 6) MCP-1 mRNA levels \(F(2, 24) = 24.426, P < 0.0005\) (Figure 5D). HMOS and GOS interacted with IL-1β induction of 1) IL-8 \(F(2, 24) = 196.011, P < 0.0005\); 2) MIP-3α \(F(2, 24) = 221.556, P < 0.0005\); 3) MCP-1 \(F(2, 24) = 144.407, P < 0.0005\) (Figure 5B); 4) IL-8 mRNA levels \(F(2, 24) = 61.374, P < 0.0005\); 5) MCP-1 mRNA levels \(F(2, 24) = 10.318, P < 0.0005\).
comparisons. Although immature enterocytes are more sensitive
and microbiological (39) research. Milk oligosaccharides were
interpreted to inflammatory stimuli than mature enterocytes, H4 inflam-
amation was as responsive to quenching by HMOS and GOS, suggesting that HMOS and GOS might inhibit inflammatory
in immature whole intestinal mucosal tissue.

Inflammatory response in human fetal intestine. Experi-
ments analogous to the in vitro studies were performed by using
organ cultures of immature human intestinal mucosa to confirm
the effects of GOS and HMOS on TNF-α–induced inflammation in normal fetal (20–22-wk gestation) human intestine ex vivo.
Basal IL-8 and MCP-1 secreted by the fetal tissues were not
significantly altered by HMOS, GOS, or cellobiose (Figure 6).
Two-factor ANOVA indicated a significant interaction between
TNF-α treatment and oligosaccharides. HMOS and GOS interacted
with TNF-α for the induction of 1) IL-8 [F (3, 32) = 48.981, P < 0.0005]; 2) MCP-1 [F (3, 32) = 240.629, P < 0.0005] (Figure 6A); 3) IL-8 mRNA levels [F (3, 32) = 65.854, P < 0.0005];
4) MCP-1 mRNA levels [F (3, 32) = 121.761, P < 0.0005] (Figure 6B).

Salmonella infected human fetal intestine. Infection of
cultured immature intestinal mucosa by Salmonella enterica
serovar Typhimurium strain SL1344 upregulated IL-8 and
MCP-1 protein and mRNA levels, P < 0.001 (Figure 7). Two-
factor ANOVA indicated a significant interaction between
Salmonella infection and oligosaccharide treatment. HMOS
and GOS interacted with Salmonella infection for the induc-
tion of IL-8 [F (3, 40) = 182.902, P < 0.0005] and MCP-1 mRNA levels
[F (3, 40) = 32.702, P < 0.0005]; 2) MCP-1 –induced IL-8 and MCP-1 to 41% and 38% of positive controls, P < 0.05
(Figure 6B). GOS attenuated TNF-α induction of IL-8 and MCP-
to 75% and 32%, respectively, of positive TNF-α–stimulated controls, P < 0.01 (Figure 6B). Cellobiose did not exhibit significant inhibition, P > 0.78 (Figure 6). These results imply that HMOS and GOS might quench the inflammatory signaling induced by
human enteric pathogens in immature human intestinal
mucosa.

Discussion
A relation between nursing and infant health was noted in
ancient times (37) and at the beginning of modern milk (38),
and microbiological (39) research. Milk oligosaccharides were

MIP-3α mRNA levels [F (2, 24) = 111.821, P < 0.0005]; 6) MCP-1 mRNA levels [F (2, 24) = 87.728, P < 0.0005] (Figure 5E). HMOS and GOS interacted with Salmonella infection of IL-8 [F (2, 24) = 868.183, P < 0.0005]; 2) MCP-1 [F (2, 24) = 351.180, P < 0.0005] (Figure 5C); 3) IL-8 mRNA levels [F (2, 24) = 84.742, P < 0.0005]; 4) MCP-1 mRNA levels [F (2, 24) = 83.510, P < 0.0005] (Figure 5F). Simple main effects analyses (Bonferroni post hoc tests) indicated that TNF-α–induced IL-8, MIP-3α, and MCP-1 and elevated their mRNA levels, P < 0.001. HMOS attenuated this induction to 48–50% of positive TNF-α controls, and GOS reduced the induction to 25–26% of TNF-α controls (Figure 5A, D). IL-1β also induced IL-8, MIP-3α, and MCP-1 protein and their mRNA levels, P < 0.001 (Figure 5B, E). HMOS attenuated this induction to 45–51% of IL-1β positive controls, and likewise GOS reduced this induction to 69–74% of positive controls, P < 0.001. Salmonella infection similarly induced IL-8 and MCP-1 secretion and their mRNA levels, P < 0.001 (Figure 5C, F). HMOS strongly attenuated IL-8 and MCP-1 induction to 26% and 30% of Salmonella-infected positive controls, and GOS decreased IL-8 and MCP-1 induction to 36% and 39% of infected positive controls, respectively, P < 0.01 (Figure 5C). Basal levels of IL-8, MIP-3α, and MCP-1 protein or mRNA were not affected by the presence or absence of GOS, HMOS, or cellobiose in H4 cells (not shown). The percentage reduction was similar for both
protein and mRNA for each pro-inflammatory mediator and all
comparisons. Although immature enterocytes are more sensitive
identified that stimulated intestinal colonization by *Bifidobacterium bifidum* (12, 39). Biological activity of HMOS and larger glycans (1, 3, 4, 6) is clinically significant (5, 6, 8) and can be categorized into 3 types. First, specific oligosaccharides inhibit specific pathogen binding to mucosal receptors, protecting breastfeeding infants (4). Second, the indigestible HMOS specifically pathogen binding to mucosal receptors, protecting breastfeeding infants (4). Second, the indigestible HMOS protects enterocytes, providing a common mechanism whereby HMOS and GOS, and TNF-α induction of IL-8 (F(2, 24) = 391.177, P < 0.0005), MIP-3α [F(2, 24) = 519.049, P < 0.0005], and MCP-1 [F(2, 24) = 503.941, P < 0.0005] (A) and IL-8 mRNA levels [F(2, 24) = 60.646, P < 0.0005], MIP-3α mRNA levels [F(2, 24) = 16.947, P < 0.0005], and MCP-1 mRNA levels [F(2, 24) = 24.426, P < 0.0005] (B). HMOS and GOS interacted with IL-1β induction of IL-8 (F(2, 24) = 196.011, P < 0.0005), MIP-3α [F(2, 24) = 221.556, P < 0.0005], and MCP-1 [F(2, 24) = 144.407, P < 0.0005] (C) (B) and IL-8 mRNA levels [F(2, 24) = 61.374, P < 0.0005], MIP-3α mRNA levels [F(2, 24) = 111.821, P < 0.0005], and MCP-1 mRNA levels [F(2, 24) = 87.728, P < 0.0005] (D). HMOS and GOS interacted with Salmonella induction of IL-8 [F(2, 24) = 686.183, P < 0.0005], MCP-1 [F(2, 24) = 361.180, P < 0.0005] (E) and IL-8 mRNA levels [F(2, 24) = 84.742, P < 0.0005] and MCP-1 mRNA levels [F(2, 24) = 83.510, P < 0.0005] (F). Simple main effects analyses (Bonferroni post hoc tests) are shown: labeled bars without a common letter differ, P < 0.01. Con, control; GOS, galactosyloligosaccharides; HMOS, human milk oligosaccharides; H4, human normal fetal intestinal epithelial cell; MCP-1, monocyte chemoattractant protein-1; MIP-3α, macrophage inflammatory protein-3α.

Intestinal epithelial T84 (transformed) and NCM-460 (non-transformed) cells were models of adult human enterocytes; both GOS and HMOS attenuated TNF-α–induced MIP-3α. TNF-α–mediated IL-8 induction was modestly but significantly inhibited by HMOS only in NCM-460, but not T84, cells (Figure 2). Lower activity of HMOS relative to GOS in T84 cells may reflect lower galactosyllactoses in HMOS (from pooled milk; ~18% colostrum) than GOS. At 70% confluence, the T84 cells may also be less polarized, potentially influencing their sensitivity to inflammation and its inhibition. Both GOS and HMOS strongly inhibited the inflammatory mediators in H4 cells (Figure 5). H4 models epithelia of human immature intestine (15, 16), with greater induction of chemokines by inflammatory insult (3, 15–17) and thus more sensitivity to inhibition than mature gut cells. HMOS and GOS significantly attenuated inflammation induced in ex vivo immature gut mucosa from 20–22 wk of gestational age (Figure 6), which is close in maturation to the most vulnerable earliest premature infants (1, 3, 17). These in vitro and ex vivo models were subjected to an additional clinically relevant insult, infection by *Salmonella* and *Listeria;* HMOS and GOS attenuate the ability of these enteric pathogens to induce chemokine induction (Figures 4, 5, and 7). The active galactosyllactoses are highest in colostrum, the form of milk that comes into immediate direct contact with the most immature enterocytes of the newborn mucosa. This implies an age-dependent physiologic pairing of colostrum with immature mucosa that permits microbial exposure to the new mucosa without causing excessive inflammation.

Enterocyte cell culture reductionist models of gut function allow signaling pathways to be studied in the absence of many confounding variables. The multiple in vitro models used in these studies were congruent in indicating that activation of NF-κB was inhibited by HMOS and GOS (Figure 3). NF-κB activation is the critical step of inflammatory signal transduction, providing a common mechanism whereby HMOS and GOS could reduce both chemokine-induced pro-inflammatory signaling and pathogen-induced inflammation.
That oligosaccharides strongly inhibit transcriptional induction of pro-inflammatory genes, but not their basal expression, is concordant with galactosyllactose modulating the NF-κB inflammatory pathway. This may be analogous to heparin or heparan sulfate disaccharides attenuating the response to pro-inflammatory stimuli in intestinal epithelial cells (40). The intestinal epithelium is a polarized unicellular barrier with its apical and basolateral surfaces normally separated by tight junctions, and these surfaces are functionally distinct in mature gut (41, 42). Neutral human milk oligosaccharides are transported into intestinal epithelium and move across the epithelial monolayer by receptor-mediated transcytosis (43). Galactosyllactose could exhibit similar putative transport and binding as part of its inhibition of inflammatory signaling. Alternatively, galactosyllactoses could alter intracellular signaling in enterocytes through binding specific cell surface receptors to attenuate NF-κB signaling, thereby quenching inflammation.

Inflammation compromises the mucosal barrier and exacerbates the translocation of bacteria and other foreign antigens into the submucosa (44), further escalating the inflammation. Necrotizing enterocolitis in premature infants is a result of unrestrained inflammation in the immature gut (2, 17, 45), and risk is directly proportional to the degree of immaturity of the gut, and inversely proportional to the degree of consumption of human milk. By reducing inflammation, both HMOS and GOS may prevent, arrest, and reverse inflammation causing necrotizing enterocolitis. The findings herein suggest that reduced risk by increased degree of human milk consumption could be partially attributed to the ability of galactosyllactose and perhaps other HMOS to quench the excessive inflammatory response of the mucosa.

Another port of entry for pathogens is M-cells (46–48). M-cells sample luminal antigens but can be used by some enteropathogens, such as Salmonella, Shigella, Listeria, and Brucella, to gain passage to the basolateral side of the polarized epithelium (33, 46, 47). Thus, the ex vivo immature human mucosa models used in this study, which allow exposure by any stimuli of both apical and basolateral aspects of the epithelial cells (analogous to the in vitro models), replicate the initial pathophysiology of Salmonella or Listeria infection of normal immature gut (33, 34, 40, 41, 49). Thus, the strength and constancy of HMOS and GOS suppression of humoral- and pathogen-induced inflammation in our models could be due to limiting NF-κB signaling secondary to infection from both the apical and basolateral surface of the epithelium.

In aggregate, galactosyllactoses found in human colostrum inhibit the NF-κB signaling pathway, thereby attenuating the intestinal epithelial response to many inflammatory stimuli, including inflammation induced by clinically relevant pathogens.

The strategy for human reproduction is large and prolonged investment by the mother into infant survival and maturation. Prolonged lactation with milk rich in protective components is fundamental to this process. We propose that the oligosaccharides are part of an innate immune system of human milk whereby the mother protects her infant. This system includes 3 major functions: competitive inhibition of binding and infection by specific pathogens, a prebiotic activity promoting colonization by mutualistic bacteria, and direct inhibition of

![FIGURE 6](image-url) **FIGURE 6** HMOS and GOS suppressed TNF-α-induced protein (A) and mRNA expression (B) of IL-8 and MCP-1 in human fetal intestine ex vivo. Chemokine secretion was expressed per total protein and normalized to TNF-α positive controls; mRNA levels are fold of negative controls (Con). All data are means ± SEMs of 3 independent experiments. Two-factor ANOVA indicated a significant interaction between TNF-α treatment and the oligosaccharides, HMOS and GOS, for the induction of IL-8 [F(3, 32) = 48.981, P < 0.0005] and MCP-1 [F(3, 32) = 240.629, P < 0.0005] (A) and IL-8 mRNA levels [F(3, 32) = 65.854, P < 0.0005] and MCP-1 mRNA levels [F(3, 32) = 121.761, P < 0.0005] (B). Simple main effects analyses (Bonferroni post hoc tests) are shown: labeled bars without a common letter differ, P < 0.05. Cello, cellobiose; Con, control; GOS, galactosyloligosaccharides; HMOS, human milk oligosaccharides; MCP-1, monocyte chemoattractant protein-1.

![FIGURE 7](image-url) **FIGURE 7** HMOS and GOS quenched Salmonella-induced protein (A) and mRNA expression (B) of IL-8 and MCP-1 in human fetal intestine ex vivo. Chemokine secretion was expressed per total protein and normalized to TNF-α positive controls; mRNA levels are fold of negative controls (Con). All data are means ± SEMs of 3 independent experiments. Two-factor ANOVA indicated a significant interaction between Salmonella infection and HMOS and GOS oligosaccharide treatment for the induction of IL-8 [F(3, 32) = 14.801, P = 0.018] and MCP-1 [F(3, 32) = 182.902, P < 0.0005] (A) and IL-8 mRNA levels [F(3, 32) = 32.702, P < 0.0005] and MCP-1 mRNA levels [F(3, 32) = 110.777, P < 0.0005] (B). Simple main effects analyses (Bonferroni post hoc tests) are shown: labeled bars without a common letter differ, P < 0.01. Cello, cellobiose; Con, control; GOS, galactosyloligosaccharides; HMOS, human milk oligosaccharides; MCP-1, monocyte chemoattractant protein-1.

Human milk galactosyllactose and gut inflammation
inflammation in the immature gut. This protection facilitates extraterine maturation of the infant mucosa. Infant development is a rapidly unfolding process with changing needs over the course of development. Human milk components, especially HMOS, also change over the course of lactation. The uniquely high occurrence of galactosyllactose in colostrum and its rapid decline over early lactation suggests a prominent role immediately after parturition in providing transient immunomodulation of the hypertensive intestinal mucosa. This facilitates the transition from the hyperresponsive fetal gut to the more accommodating quiescent gut of the colonized mucosa. Thus, galactosyllactose promotes mutualistic harmony between the incipient microbiota and its human host through direct inhibition of inflammation, as demonstrated herein. Dietary supplementation with GOS may prove useful as a potential prophylactic and therapeutic agent against enteric inflammation, especially the inflammation common to many neonatal disease states of diverse etiology.

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