Applications of biophysical methods in small-molecule modulators targeting protein function

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Chapter 7

The human milk oligosaccharides 3-FL, LNnT, and LDFT attenuate TNF-α induced inflammation in fetal intestinal epithelial cells through shedding or interacting with TNF receptor 1

In submission

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Abstract

Human milk oligosaccharides (hMOs) can attenuate systemic and intestinal inflammation by modulating intestine epithelial cells, but the mechanisms of action are not well-understood. Here we study the effects of six different hMOs (2'-FL, 3-FL, 6'-SL, LNT, LNnT, LDFT) and one hMOs acid hydrolysate LNT2 on TNF-α induced inflammatory event in immature and mature gut epithelial cells, and we explore the possible mechanisms of action. 3'-FL, LNnT, and LDFT significantly attenuated TNF-α induced inflammation in immature intestine epithelial cells, while LNT2 induced IL-8 secretion in mature intestine epithelial cells. The anti-inflammatory effects of 3'-FL, LNnT, and LDFT were through TNFR1-signalling inhibition through different mechanisms. 3'-FL, LNnT, and LDFT exerted TNFR1 ectodomain shedding while LNnT also showed binding affinity to TNFR1.

Keywords

human milk oligosaccharides; TNF-α; intestine cells; inflammation; TNF receptors.

Introduction

Breastfeeding is the gold standard for infant nutrition as it offers complete nutrition and essential bioactive components for the development of the newborn (Doare, Holder, Bassett, Pannaraj, 2018). Exclusive breastfeeding is therefore recommended for the first six months of life by the World Health Organization (WHO) (Walker, 2010). For a variety of reasons, over 70% of the infants can not be exclusively breastfed (Heymann, Raub, Earle, 2013), as a consequence of which the infants have to be fed with cow-milk based infant formula, which attempts to mimic the nutritional composition of breast milk (Coulet, Phothirath, Allais, Schilter, 2014; Piemontese *et al.*, 2011). However, currently, these infant formulas do not contain the same bioactive molecules as human milk (Aly, Ali Darwish, Lopez-Nicolas, Frontela-Saseta, Ros-Berruezo, 2018), as a consequence

these formula-fed babies have a higher risk of infections and inflammatory diseases than babies solely fed with infant formula (Ladomenou, Moschandreas, Kafatos, Tselentis, Galanakis, 2010). One of the most important bioactive components of mother milk are human milk oligosaccharides (hMOs), which are unique to humans and are not found in the same variety and composition in other mammals (Bode, 2012). Recently, major advances have been made with the inclusion of human milk oligosaccharides (hMOs) in cow-milk based infant formula. Some hMOs can be produced via genetically engineered microorganisms and are now applied in infant formulas (Vandenplas *et al.*, 2018).

It has been shown that hMOs provide multiple health-promoting effects, which include support of growth of beneficial bacteria (Asakuma *et al.*, 2011), anti-pathogenic effects (Morrow *et al.*, 2004), immune modulating effects (Cheng, Kiewiet, Groeneveld, Nauta, de Vos, 2019), enhancement of intestinal barrier function (Cheng, Kong, Walvoort, Faas, de Vos, 2019; Wu *et al.*, 2019), as well as attenuation of systemic and intestinal inflammation (Y. He, Liu, Leone, Newburg, 2014). It is however still unclear which and how individual hMOs contribute to processes such as prevention and attenuation of intestinal inflammatory events (Bode *et al.*, 2016). It has been reported that hMO can directly interact with intestinal cells and modulate immunity (Y. Y. He *et al.*, 2016). The majority of hMOs can reach the intestine without being digested and some undergo hydrolyzation at low pH during transit through the gastrointestinal tract (Gnoth, Kunz, Kinne-Saffran, Rudloff, 2000). This may lead to the formation of lacto-N-triose II(LNT2), which is the acid hydrolysate of the tetra and higher hMOs such as lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT) (Bidart, Rodríguez-Díaz, Yebra, 2016; Gnoth *et al.*, 2000). How these acid hydrolysates impact the inflammatory responses through intestine epithelial cells is also not known.

HMOs are considered to guide immune development during early postnatal intestinal gut immune barrier development (Figueroa-Lozano, de Vos, 2019; Wu *et al.*, 2019). This early postnatal developing gut is very susceptible for inflammatory events (Yu *et al.*, 2018). Disturbances in intestinal immune development in neonates may lead to intestinal inflammatory diseases such as necrotizing enterocolitis (NEC) and inflammatory bowel diseases (IBD) (Schirbel, Fiocchi, 2011; Wu *et al.*, 2019). Tumor necrosis factor-α (TNF-α) plays an important role in these inflammatory diseases (Bradley, 2008). To

induce inflammation, TNF-α needs to bind to the TNF-receptors on the cell surface. There are two different receptors for TNF-α, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (Bradley, 2008). These receptors have two distinct roles, TNFR1 mainly induces inflammatory signaling pathways, while TNFR2 mediates immune modulatory functions and promotes tissue homeostasis and regeneration (Bradley, 2008). Therefore, the blockage of TNFR1 signaling, either by binding with TNFR1 as antagonists or by increasing the shedding of soluble TNFR1, may attenuate the response of the cells to TNF-α (Fischer, Kontermann, Maier, 2015). Whether hMOs interfere with these TNFR signaling pathways is subject of investigation in the current study.

In the present study, we investigated the effects of six different hMOs (2'-FL, 3-FL, 6'-SL, LNT, LNnT, LDFT) and one hMOs acid hydrolysate LNT2 on TNF-α induced inflammatory event in gut epithelial cells. To this end, two types of gut epithelial cells were tested. An immature human primary fetal intestinal epithelial cell FHs 74 Int was applied as well as the adult colonic epithelial cell line T84 cells (Y. Y. He *et al.*, 2016) to determine possible differences in the efficacy of hMOs in modulating inflammatory events in immature or adult cells. As the IL-8 induction by intestine epithelial cells strongly reflects the degree of inflammatory response after stimuli (Nanthakumar *et al.*, 2011), IL-8 secretion in FHs 74 Int and T84 were measured to determine possible hMO induced attenuation of inflammatory events. In order to further explore the possible mechanisms of action, the interactions between effective hMOs and TNFR1 were also investigated, which including the possible binding affinity between hMOs and TNFR1, and whether hMOs cause TNFR1 ectodomain shedding.

Hypotheses

Human milk oligosaccharides attenuate TNF-α induced inflammation through TNFR1 in a hMO structure-dependent way.

Material and Method

Components

In the present study, 2'-FL (provided by FrieslandCampina Domo, Amersfoort, the Netherlands), 3-FL, 6'-SL, LNT2, LNT, LNnT, and LDFT (provided by Glycosyn LLC, Woburn, MA, USA) were tested. An overview of the structure and components are shown in Table 1.

Cell culture and reagents

Nontransformed human small intestinal epithelial FHs 74 Int cells (ATCC, Manassas, VA, USA) were maintained in Hybri-Care medium (ATCC, Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma–Aldrich, Zwijndrecht, The Netherlands), 50μg/mL Penicillin-Streptomycin Solution (Sigma–Aldrich, Zwijndrecht, The Netherlands), and 30ng/ml EGF (ATCC, Manassas, VA, USA). Human colon carcinoma T84 cells were cultured in Dulbecco's Modified Eagle Medium:F-12 (DMEM:F12) medium (Gibco, Life Technologies, Bleiswijk, The Netherlands) supplemented with 10% heat-inactivated fetal bovine serum (Sigma–Aldrich, Zwijndrecht, The Netherlands) and 50 mg/mL gentamicin (Lonza, Verviers, Belgium). Cells were cultured at 37°C in 5% CO_2 as recommended by the manufacturer. Recombinant human TNF-α was obtained from PeproTech (Rocky Hill, NJ, USA).

Cell viability and WST-1 assay

FHs 74 Int and T84 cells were resuspended in fresh culture medium at 10,000cells/mL, after which 200μL of cell suspension was seeded per well in 96-well plates (Corning, NY, USA). Cells were then cultured until reaching 70–80% confluence. Prior to treatment, cells were washed twice with phosphate-buffered saline (PBS; Lonza, Verviers, Belgium), after which, culture medium was replaced by 100μL of fresh medium containing one of the ingredients. FHs 74 Int and T84 cells treated with 5mg/mL of 2'-FL, 3-FL, 6'-SL, LNT2, LNT, LNnT, and LDFT for 24h. Cell viability was determined by WST-1 assay following the manufacturer's instructions. Briefly, after 24h treatment, 10μL WST-1 reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to the culture medium in 1:10 final dilution and incubated for 1h at 37°C and 5% CO_2. Absorbance (450nm) was measured using a Benchmark Plus Microplate Reader using Microplate Manager version 5.2.1 for data acquisition. The data for each sample was plotted as the percentage change compared to the negative control.

Cell stimulation

FHs 74 Int and T84 cells were resuspended and seeded at 10,000 cells/mL in a flat bottom 96-well plate at 200μL per well. Cells were then cultured until reaching 70–80% confluence. Prior to treatment, cells were washed twice with phosphate-buffered saline (PBS; Lonza, Verviers, Belgium), after which culture medium was replaced by 200μL of fresh medium containing one of the ingredients. For IL-8 induction, FHs 74 Int and T84 cells were treated with 5mg/mL of 2'-FL, 3-FL, 6'-SL, LNT2, LNT, LNnT, and LDFT in the absence or presence of 10ng/mL TNFα for 24h. After 24h of incubation, the secretion of the proinflammatory cytokine IL-8 was measured in the supernatant by ELISA (R&D SYSTEM, Minneapolis, MN, USA) according to the manufacturer's protocol. For soluble TNFR1 measurement, FHs 74 Int cells were treated with 5mg/mL of 3-FL, LNnT, and LDFT in the absence or presence of 10ng/mL TNF-α for 24h. After 24h of incubation, the soluble TNFR1 was measured in the supernatant by ELISA (R&D SYSTEM, Minneapolis, MN, USA) according to the manufacturer's protocol.

Western blot

FHs 74 Int and T84 cells were resuspended and seeded at 10,000 cells/mL in a 6 wells plate at 2mL per well for 48h. After that, the culture medium was replaced by 2mL of fresh medium in the absence or presence of 10ng/mL TNF-α for 24h. After the treatments, total protein extracts were obtained from cells. Cells were harvested in ice-cold PBS and lysed with RIPA Lysis and Extraction Buffer (Thermo Scientific, MA, USA) supplemented with Protease Inhibitor Cocktails (Sigma-Aldrich, Zwijndrecht, The Netherlands). Lysates were sonicated (5s twice) and centrifuged (12000g, 20min, 4°C). Subsequently the supernatants were collected, and protein yield was quantified using Pierce BCA Protein Assay Kit (Thermo Scientific, MA, USA). Normalized 30μg of protein samples were prepared with Laemmli sample buffer containing β-mercaptoethanol and electrophoresed on an SDS-polyacrylamide gel. Proteins were transferred to a PVDF membrane (Sigma-Aldrich, Zwijndrecht, The Netherlands). After blocking for 1h with 1:1 mixture of Licor blocking buffer (LI-COR Biosciences) and 1x PBS for the blocking solution, the membrane was probed with the primary antibody TNFR1 (1:500, Abcam, Cambridge, UK) and TNFR2 (1:1000, Abcam, Cambridge, UK) overnight at 4°C. After that, the membrane was washed in 4x PBS-T and followed by incubation with the secondary antibody for 1h at room temperature. Immunoreactivity was visualized by the Odyssey Imaging System (LI-COR Biosciences). Signal intensity was analyzed by using Image J (National Institutes of Health, Bethesda, MD).

TNFR1 binding assay

The binding affinity between hMOs and TNFR1 was quantified with the microscale thermophoresis (MST) assay. MST experiments were performed on a NanoTemper ® Monolith NT.115 with blue/red filter (NanoTemper Technologies GmbH, Munich, Germany) according to the manufacturer's protocol. After optimization, the final concentration of His-tagged TNFR1 (Abcam, Cambridge, UK) was kept constant at 50nM, 10μL of the 5mM 3-FL, 5mM LDFT, and 250μM LNnT were diluted 1:1 in 10μL PBS-T buffer (PBS 1x ; 0.05% Tween20) to make a 16-sample dilution series. The 50nM His-tagged TNFR1 was incubated with dye for 30min, after that, 16 different concentrations of

samples were incubated with 50nM His-tagged TNFR1 with dye for 2h. Pre-incubated samples and protein mixtures were loaded into standard capillaries (NanoTemper Technologies GmbH, Munich, Germany), measurements were performed at 25°C using 40% MST power with 80% excitation power. All experiments were repeated at least three times. Data analyses were performed using the NanoTemper® analysis software.

Statistical analysis

The results were analyzed using GraphPad Prism. Normal distribution of the data was confirmed using the Kolmogorov-Smirnov test. Parametric data are expressed as mean standard deviation (SD). Statistical comparisons of parametric distributed data were performed using one-way ANOVA with Dunnett multiple comparison tests or two-way ANOVA for group analysis. $p < 0.05$ was considered as statistically significant (, * $p < 0.05$, , **$p < 0.01$, , ***$p < 0.001$, , ****$p < 0.0001$).

Results

hMOs and hMO's acid hydrolysis product did not influence cell viability

To exclude toxic effects of the tested hMOs on cell viability of FHs 74 Int and the human colon carcinoma cell line T84, cells were treated with 5mg/mL of 2'-FL, 3-FL, 6'-SL, LNT2, LNT, LNnT, and LDFT for 24h after which the cell viability was quantified. As shown in Figure 1, 5mg/mL of 2'-FL, 3-FL, 6'-SL, LNT2, LNT, LNnT, and LDFT did not have a statistically significant negative effect on the cell viability of both FHs 74 Int and T84 cells.

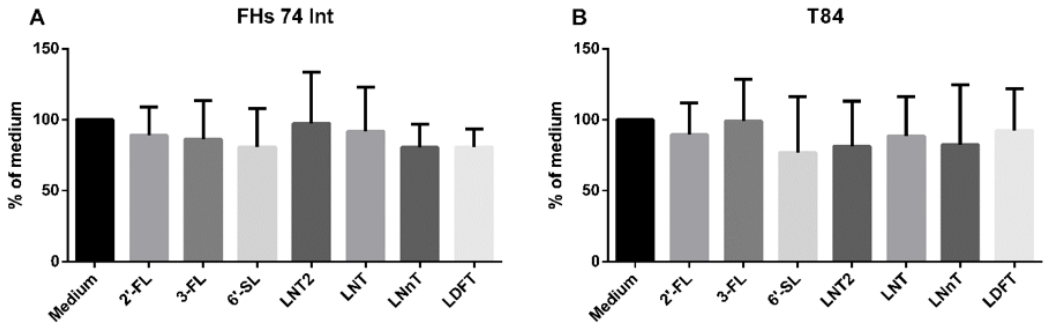


Figure 1. hMOs and the hMOs acid hydrolysate LNT2 did not alter cell viability of FHs 74 Int and T84. (A) FHs 74 Int and (B) T84 cells were treated with 5mg/mL of 2'-FL, 3-FL, 6'-SL, LNT2, LNT, LNnT, and LDFT for 24h, cells treated with culture medium served as negative control. Results are presented as percentage change against negative control. Data are presented as mean SD (n=5), significant differences compared to the negative control were determined by using one-way analysis of variance with Dunnett multiple comparisons test and indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or by **** ($p < 0.0001$).

hMO's acid hydrolysis LNT2 induces IL-8 production in T84 cells

First, the impact of the tested hMOs and the hMO's acid hydrolysis LNT2 on IL-8 production was tested as IL-8 is one of the primary gut-epithelial cell derived chemokine responsible for inducing inflammation (Cotton et al., 2016). To this end, FHs 74 Int and T84 were treated with 5mg/mL of 2'-FL, 3-FL, 6'-SL, LNT2, LNT, LNnT, and LDFT for 24h.

As shown in Figure 2A, hMOs and LNT2 were not able to modulate IL-8 secretion in the fetal cell line FHs 74 Int. Also, in T84 the tested hMOs did not alter IL-8 production but the hMO's acid hydrolysis product LNT2 significantly induced IL-8 secretion in T84 cells (Figure 2B). LNT2 significantly increased IL-8 secretion to 1.4-fold compared to the untreated control ($p < 0.0001$).

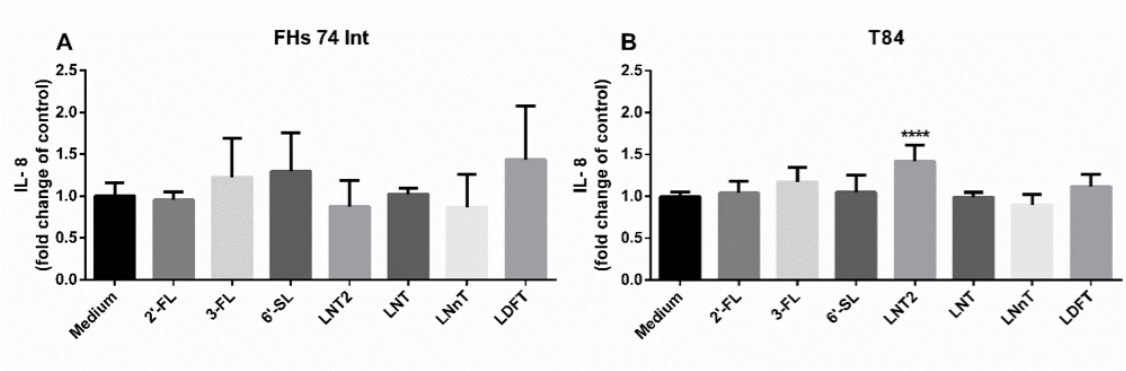


Figure 2. hMO's acid hydrolysate LNT2 induce IL-8 production in T84 cells under homeostatic conditions. FHs 74 Int (A) and T84 cells (B) were incubated with 5mg/mL of 2'-FL, 3-FL, 6'-SL, LNT2, LNT, LNnT, and LDFT for 24h. Cells treated with culture medium served as negative control. Results are presented as fold change against negative control. Data are presented as mean SD (n=6), significant differences compared to the negative control were determined by using one-way analysis of variance with Dunnett multiple comparisons test and indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or by **** ($p < 0.0001$).

hMOs attenuates TNF-α-induced IL-8 secretion in FHs 74 Int in a chemical structure-dependent way

In order to investigate the ability of hMOs and LNT2 to attenuate inflammatory responses in fetal and adult intestine epithelial cells, we investigated the effects of hMOs and LNT2 on IL-8 secretion after exposure of the epithelial cells to the proinflammatory cytokine TNF-α. To this end, FHs 74 Int and T84 cells were treated with 10ng/mL TNF-α for 24h with 5mg/mL of either 2'-FL, 3-FL, 6'-SL, LNT2, LNT, LNnT, LDFT. After that, the concentration of the proinflammatory cytokine IL-8 in the supernatant was measured. As shown in Figure 3, TNF-α significantly increased IL-8 secretion in both FHs 74 Int and T84 ($p < 0.0001$). Interestingly, the hMO's inhibiting effects on TNF-α induced IL-8-secretion was restricted to the fetal FHs 74 Int cells. 3-FL, LNnT, and LDFT reduced TNF-α induced IL-8-secretion with 70% ($p < 0.05$), 38% ($p < 0.0001$), and 64% ($p < 0.01$), respectively (Figure 3A). The HMOs 2'-FL, 6'-SL, LNT and the hydrolysis product LNT2 did not reduce the TNF-α induced IL-8 secretion. Results were different with the adult gut epithelial cell line. With the adult cell line T84, only the hMO's acid hydrolysis product LNT2 impacted TNF-α induced IL-8-secretion but this was an enhancement instead of an attenuation ($p < 0.001$).

The above results suggest that hMOs were able to suppress TNF-α induced IL-8-secretion in the fetal gut epithelial cell FHs 74 Int in a structural-dependent way, and it also confirmed that hMOs and LNT2 have different regulatory patterns on fetal cells and adult cells.

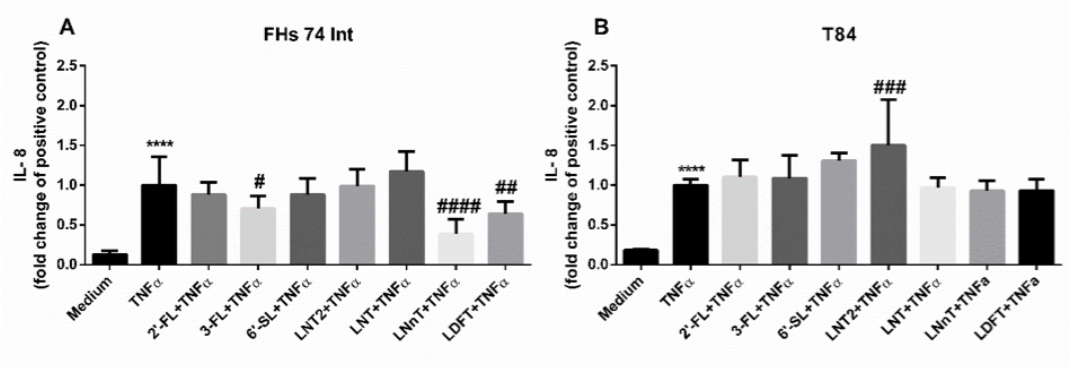


Figure 3. 3-FL, LNnT, and LDFT attenuate TNF-α-induced IL-8 secretion in FHs 74 Int cells. (A) FHs 74 Int and (B) T84 cells were stimulated with 5 mg/mL of 2'-FL, 3-FL, 6'-SL, LNT2, LNT, LNnT, and LDFT in presence of TNF-α (10 ng/mL) for 24 h. Cells treated with culture medium served as negative control. Results are presented as fold change against positive control. Data are presented as mean SD (n=6), statistical significance was analyzed using one-way analysis of variance with Dunnett multiple comparisons test (* vs. Medium; vs. TNF; , *$p < 0.05$; , **$p < 0.01$; , ***$p < 0.001$; , **** $p < 0.0001$).

FHs 74 Int and T84 have different expression patterns of TNF-α receptors

As 3-FL, LNnT, and LDFT attenuate TNF-α induced IL-8 secretion on fetal cells, we hypothesized that hMOs might have anti-inflammatory effects on fetal intestine epithelial cells through interfering in TNF-α induced proinflammatory pathway. TNF-α has two distinct cell surface receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). To determine whether the receptors are differently expressed in FHs 74 Int and T84, we compared the expression level of TNFR1 and TNFR2 after western blot. To this end, FHs 74 Int and T84 were cultured in the presence TNF-α for 24h. Cells cultured in normal medium served as controls. As shown in Figure 4A, FHs 74 Int and T84 showed different protein expression patterns of TNFR1 and TNFR2 but was not influenced by TNF-α. The protein expression of TNFR1 in fetal cells was significantly higher than in the adult cell line T84 ($p < 0.0001$, Figure 4B). However, the expression of TNFR2 in

fetal FHs 74 Int cells was significantly lower than in T84 ($p < 0.001$, Figure 4C). Neither TNFR1 nor TNFR2 was significantly altered by TNF-α (Figure 4B&C).

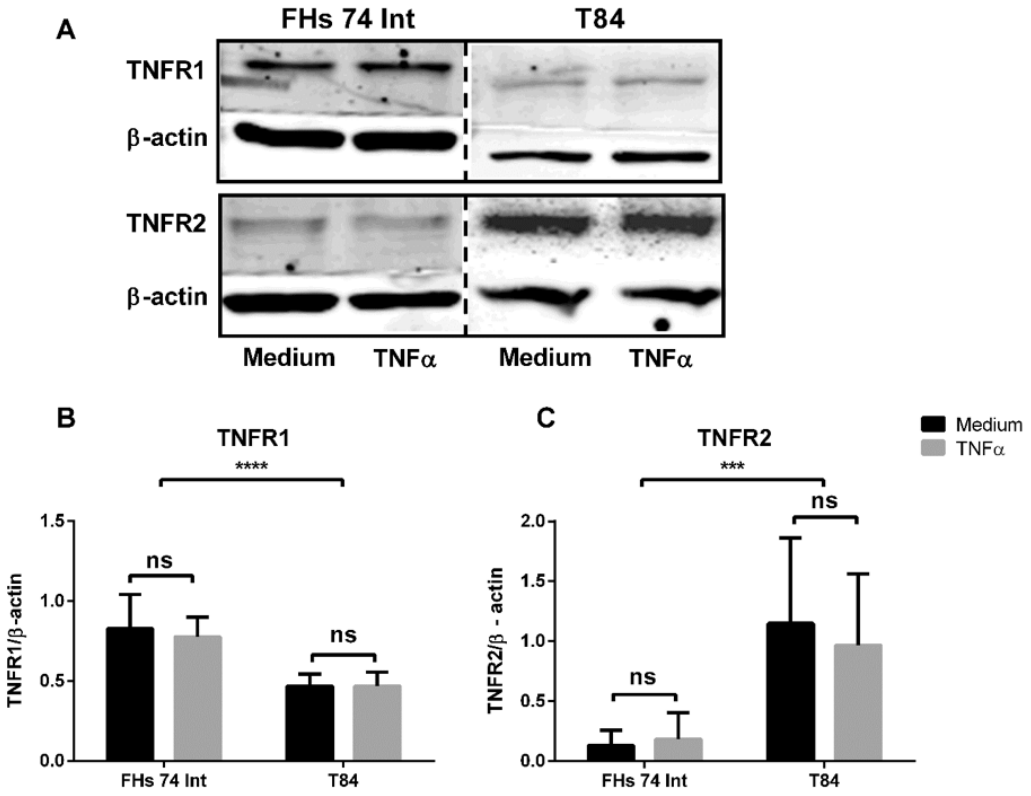


Figure 4. FHs 74 Int and T84 showed different expression pattern of TNFR1 and TNFR2. FHs 74 Int and T84 were incubated with or without 10ng/mL TNF-α for 24h. (A) The TNFR1 and TNFR2 expression in western blot. Western blot results were analyzed by using Image J gradation analysis of (B) TNFR1 and (C) TNFR2. Results are represented as mean SD (n=5). Significant differences compared between medium and TNF-α, FHs 74 Int and T84 were determined by using two-way ANOVA and indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or by **** ($p < 0.0001$).

LNnT attenuate TNF-α-induced IL-8 secretion by interacting with the TNF receptor 1 in fetal gut epithelial cells

As 3-FL, LNnT, and LDFT only inhibited TNF-α induced IL-8 secretion in the fetal cell line FHs 74 Int, and as FHs 74 Int has high expression of TNFR1 and low expression of TNFR2 compared to T84, we hypothesized that hMOs might have its anti-inflammatory effects through interaction with TNFR1. To study the interaction with hMOs and TNFR1, the binding affinity of 3-FL, LNnT, LDFT with TNFR1 was determined with microscale thermophoresis (MST). A wide concentration range of 3-FL

(0.00061μM to 5mM), LNnT (0.00763μM to 250μM), and LDFT (0.00061μM to 5mM) were incubated with His-tagged TNFR1 in a constant range (50nM) at room temperature for 2h. Subsequently the binding affinity was measured by MST. As shown in Figure 5, a ligand-dependent binding effect was detected but only LNnT interacts with TNFR1. There was no detectable binding of 3-FL and LDFT to TNFR1 (Figure 5A and C). LNnT was shown to bind TNFR1 with a Kd of 900±660nM (Figure 5B).

The above results confirm binding of LNnT to TNFR1 but also suggests that 3-FL and LDFT inhibit TNF-α induced IL-8 secretion via another mechanisms.

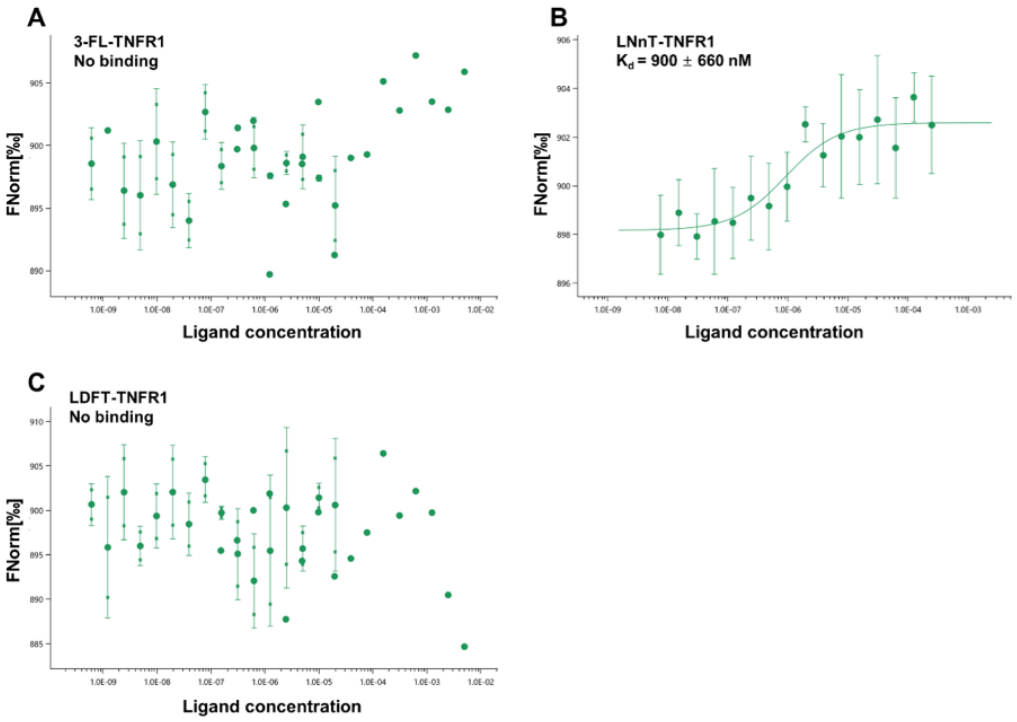


Figure 5. LNnT has binding affinity to TNFR1. Dose-response curve for the binding interaction between (A) 3-FL, (B) LNnT, (C) LDFT and TNFR1. Values on the X-axis represent the ligand (3-FL, LNnT, and LDFT) concentration, Y-axis represent the normalized fluorescence. The binding affinity is observed for the LNnT-TNFR1 interaction, which was a *Kd* of 900±660nM. All binding curves were determined in at least triplicate by MST, represented as the mean± SD.

3-FL, LNnT, and LDFT cause ectodomain shedding of TNFR1 and thereby inhibit TNF-α induced inflammation

Another possible explanation for attenuation of TNF-αinduced IL-8 secretion by 3-FL, LNnT, and LDFT is ectodomain shedding of TNFR1. TNF-α needs to bind to the receptors on the cell surface to induce downstream pro-inflammatory effects in epithelial cells (Dostert, Grusdat, Letellier, & Brenner, 2019). Ectodomain shedding is a process in which the ectodomain of TNFR1 is detached from the cell-source (Bartsch *et al.*, 2010). This can be done by several mechanism and might be influence by bioactive molecules (Yang, Moon, Lee, & Park, 2016), such as hMOs. Shedding will reduce the number of receptors on the cell surface and might serve as soluble decoy protein that competes with cell-surface bound TNFR1 thereby decreasing the response of the cells to TNF-α (Bartsch *et al.*, 2010). The quantification of the soluble receptor is a measure for the degree of shedding of the ectodomain of TNFR1 (Yang *et al.*, 2016). To test whether 3-FL, LNnT, and LDFT induce ectodomain shedding of TNFR1 on FHs 74 Int, we incubated FHs 74 Int in the absence or presence of 10 ng/mL TNF-α for 24h with either 5mg/mL of 3-FL, LNnT, and LDFT. After that, the concentration of TNFR1 in the supernatant was measured. As shown in Figure 6A, 3-FL, LNnT, and LDFT did not modulate the soluble TNFR1 under homeostatic conditions. As expected, TNF-α-exposure induced a significant decrease of soluble TNFR1 ($p < 0.001$), while 3-FL ($p < 0.05$), LNnT ($p < 0.0001$), and LDFT ($p < 0.01$) significantly prevented this TNF-α induced TNFR1 concentration increase in the medium (Figure 6B). TNF-α inhibited TNFR1 ectodomain shedding to 70% compared to the medium control ($p < 0.001$), and treatment with 3-FL, LNnT, and LDFT restored TNFR1 to 87% ($p < 0.05$), 108 percent ($p < 0.0001$), and 92% ($p < 0.01$) respectively. This suggests that 3-FL, LNnT, and LDFT could attenuate TNF-α induced inflammation by TNFR1 ectodomain shedding in fetal cell line FHs 74 Int.

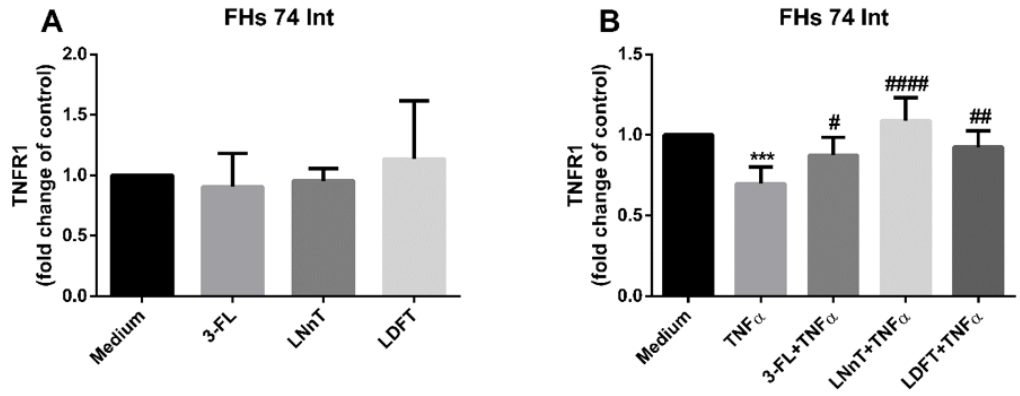


Figure 6. hMOs cause ectodomain shedding of TNFR1. FHs 74 Int cells were incubated with 5 mg/mL of 3-FL, LNnT, LDFT in the absence (A) or presence (B) of 10 ng/mL TNF-α for 24h. Cells treated with culture medium served as negative control. Data are presented as mean SD (n=5), statistical significance was measured using one-way analysis of variance with Dunnett multiple comparisons test (* vs. Medium; vs. TNF; , *p $<$ 0.05; , **p $<$ 0.01; , ***p $<$ 0.001; , **** p $<$0.0001).

Discussion

Previous studies have shown that hMOs in human milk can attenuate intestinal inflammation (Y. He *et al.*, 2014), however, how individual hMO's impact inflammatory responses in intestinal epithelial cells and which mechanisms are involved is still largely unknown. Here we studied, to the best of our knowledge for the first time, the attenuating effects of six different hMOs and one hMOs acid hydrolysate on TNF-α induced inflammatory responses in fetal and adult intestinal epithelial cells. TNF-α is a key-cytokine in necrotizing enterocolitis (NEC) and inflammatory bowel diseases (IBD) (Bradley, 2008). We show that the modulatory effects of individual hMOs are strongly structure-dependent and that attenuating effects on inflammatory responses are mainly observed in immature fetal epithelial cells that express more TNFR1. Especially 3'-FL, LNnT, and LDFT significantly attenuated TNF-α induced IL-8 secretion in fetal cells FHs 74 Int. The anti-inflammatory effects of effective hMOs were strongly related to TNFR1 through different mechanisms, 3'-FL, LNnT, and LDFT exerted TNFR1 ectodomain shedding while LNnT showed binding affinity to TNFR1.

As Kuntz *et al.* reported that acidic and neutral hMOs isolated from human milk may impact the viability of intestinal epithelial cells under homeostatic conditions (Kuntz,

Rudloff, & Kunz, 2008) and thereby decrease cell-responses such as release of cytokines. To exclude such an impact on viability and IL-8 secretion in our study we tested the impact of the hMOs on viability of FHs 74 Int and T84 cells and confirmed that the tested hMOs had no negatively impact on cell-survival in the concentration range applied. Also, we confirmed that under homeostatic condition in the absence of an inflammatory challenge, the six tested hMOs did not alter the IL-8 secretion. Surprisingly we found that only the hMOs acid hydrolysate LNT2 increased IL-8 secretion in T84 cells under homeostatic condition but also under TNF-α stimulation. This might be explained by the strong activation effects of LNT2 on Toll-like receptors (TLRs) signaling (Cheng, Kiewiet, *et al.*, 2019). Cheng *et al.* showed that LNT2 significantly activated TLRs signaling and induced cytokine production in THP-1 macrophages (Cheng, Kiewiet, *et al.*, 2019). T84 constitutively express TLRs (Cario *et al.*, 2000; Melmed *et al.*, 2003), which could be responsible for the increased production of the proinflammatory cytokine IL-8 when exposed to LNT2.

3-FL, LNnT, and LDFT attenuated TNF-α induced IL-8 secretion in immature epithelial cells, while 2'-FL, 6'-SL, and LNT did not change the inflammatory response. The effective 3-FL and non-effective 2'-FL only differ in the attachment position of L-fucose (Fuc) residues on the lactose core region, while the effective hMO LNnT and non-effective LNT differ in their hMO type 1 chain (Galβ1-3GlcNAc-) or type 2 chain (Galβ1-4GlcNAc-) linkage on lactose (Table 1). This underpins our previous notion that seemingly minor differences in molecular structure of the molecules can have significant impact on their biological actions (Cheng, Kiewiet, *et al.*, 2019). As effects were related to attenuation of the effects of TNF-α, we focused on possible interactions of the hMOs with the two receptors for TNF-α. The immature intestinal epithelial cell FHs 74 Int expressed more of the proinflammatory variant, i.e. TNFR1 than T84, while the more immune regulatory receptor TNFR2 (Bradley, 2008) was lower in FHs 74 Int than in T84 (Figure 4). This might explain the more pronounced impact of hMOs on the immature fetal cells than on the adult cells and the higher susceptibility of fetal cells for inflammation (Rognum, Thrane, Stoltenberg, Vege, & Brandtzaeg, 1992). *In vivo*, most of the proinflammatory effects of TNF-α are exerted via TNFR1 (Figure 7A) (Horiuchi, Mitoma, Harashima, Tsukamoto, & Shimoda, 2010). As shown in our current study 3-FL, LNnT, and LDFT all attenuated TNF-α induced inflammation by interfering with

this TNF-α binding to cell-surface bound TNFR1. During TNF-α stimulation, 3'-FL, LNnT, and LDFT could induce shedding of the TNFR1 ectodomain which subsequently serve as soluble decoy protein that competes with cell-surface bound TNFR1 as well as by decreasing the number of cell-surface bound TNFR1 available for ligand binding. This shedding process involves the proteolytic cleavage of TNFR1 ectodomains, which is dependent on the activation of TNF-converting enzyme (TACE) (Steeland, Libert, & Vandenbroucke, 2018). This process is, as shown here, might be stimulated by 3'-FL, LNnT, and LDFT and thereby contributes to lowering of inflammatory events in immature epithelial cells (Figure 7B). Besides induced ectodomain shedding of TNFR1, LNnT could also bind TNFR1 which inhibited the TNF-α/TNFR1 signaling pathway (Figure 7C). The multifold ways of inhibition of LNnT coincides with the observation that LNnT had a stronger inhibiting effect than 3-FL and LDFT on TNF-α induced IL-8 secretion.

In the present study, we provide evidence that some hMOs attenuate TNF-α induced inflammation by directly influencing TNFR1 signaling, and subsequently inhibiting inflammatory responses. It therewith might also be instrumental in preventing or attenuating inflammatory events in TNF-α dependent diseases such as rheumatoid arthritis and inflammatory bowel disease (Steeland *et al.*, 2018). Currently, these diseases are treated with TNF-α-inhibitors that block both TNFR1 and TNFR2, and not only TNFR1 signaling which is the main proinflammatory receptor (Steeland *et al.*, 2018). This is undesired as TNFR2 is involved in tissue repair and immune modulation and should therefore not be suppressed (Fischer *et al.*, 2015). Here we show that specific chemical hMO structures may specifically block TNFR1 signaling which might be beneficial for the treatment of the aforementioned diseases (Steeland *et al.*, 2018).

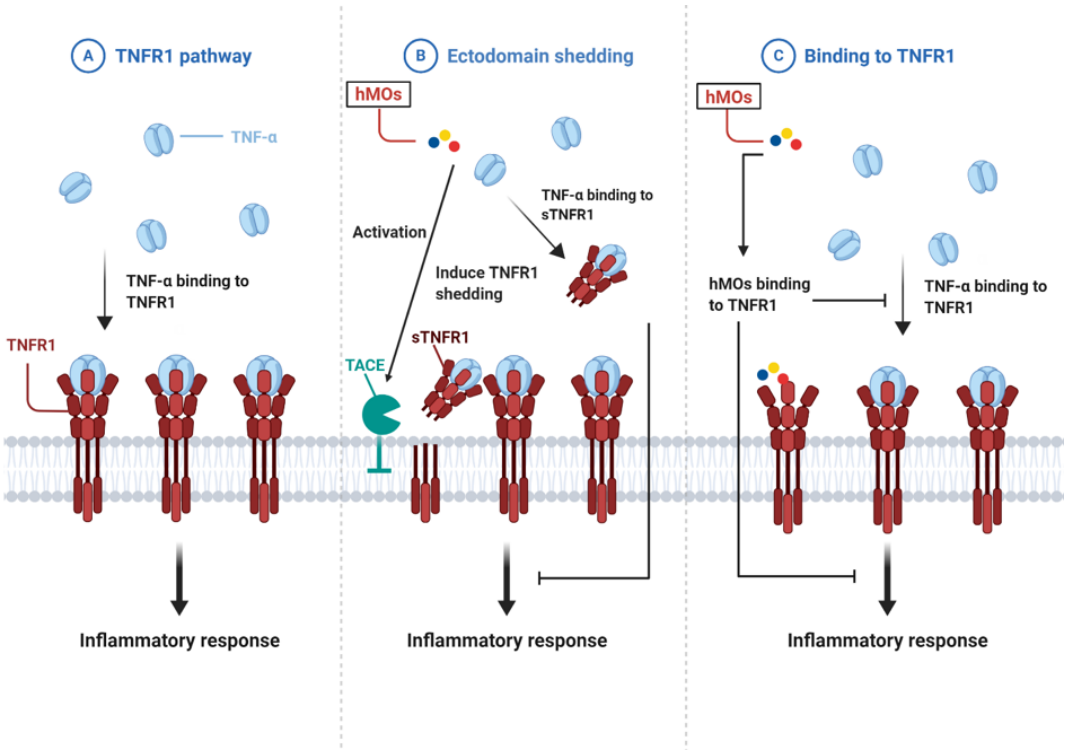


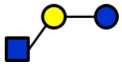
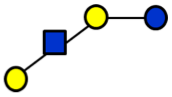
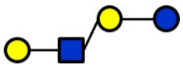
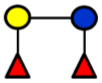
Figure 7. Schematic representation of the mechanisms of action of hMOs attenuate TNF-α induced inflammation via TNFR1. (A) TNF-α signaling via its receptors TNFR1; (B) hMOs induce shedding of cell-surface TNFR1 through activating TNF-converting enzyme (TACE), increase soluble TNFR1 (sTNFR1) and inhibit the inflammatory response; (C) hMOs binding to TNFR1, inhibit the binding of TNF-α and inflammatory response.

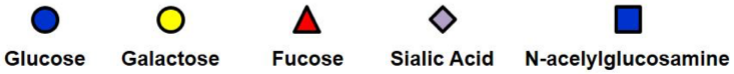
Conclusions

In conclusion, we demonstrate that specific hMO types inhibit TNF-α induced inflammatory responses in fetal gut epithelial cells in a structure-dependent fashion. Especially 3-FL, LNnT, and LDFT can effectively attenuate TNF-α induced inflammation by interacting with the TNFR1 receptor which is highly expressed in the fetal cells compared to adult gut epithelial cells. Our findings not only contribute to better understanding of the structure-function relationship of hMOs, but opens new venues to explore hMOs in management of TNF-α dependent diseases as the hMOs have more specificity for the proinflammatory pathways than currently applied TNF-α-inhibitors. Understanding how and which hMOs have anti-inflammatory effects could contribute to the future design of hMO containing products with predictable beneficial effects in specific target groups. A possible application of the current knowledge is application of 3-FL, LNnT, and LDFT in infant formula for premature neonates that are more prone to NEC or other inflammatory

disorders than term born babies.

Table 1. Overview of the structure of selected hMOs.

Name (abbreviated)	Structure	Schematic diagram
2'-FL	Fucα1-2Galβ1-4Glc	
3-FL	Galβ1-4Glc Fucα1-3/	
6'-SL	NeuNAcα2-6Galβ1-4Glc	
LNT2	GlcNAcβ1-3Galβ1-4Glc	
LNT	Galβ1-3GlcNacβ1-3Galβ1-4Glc	
LNnT	Galβ1-4GlcNacβ1-3Galβ1-4Glc	
LDFT	Fucα1-2Galβ1-4Glc Fucα1-3/	



Contributions

Wenjia Wang helped to performed and analysed MST experiments.

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