Food and Nutrition Sciences, 2014, 5, 1387-1398
Published Online August 2014 in SciRes. http://www.scirp.org/journal/fns
http://dx.doi.org/10.4236/fns.2014.514151

Human Milk Oligosaccharides Enhance Innate Immunity to Respiratory Syncytial Virus and Influenza *in Vitro*

Geralyn Duska-McEwen[1], Albert P. Senft[2], Teah L. Ruetschilling[2], Edward G. Barrett[2], Rachael H. Buck[1]

[1]Discovery R & D, Abbott Nutrition, Columbus, USA
[2]Lovelace Respiratory Research Institute, Albuquerque, USA
Email: rachael.buck@abbott.com

Received 21 May 2014; revised 27 June 2014; accepted 8 July 2014

Abstract

Human milk oligosaccharides (HMO) contribute to innate immunity by enhancing growth of beneficial bacteria, epithelial cell maturation and mucosal barrier integrity. They have immunomodulatory effects and can block pathogen binding to host cell surface glycans or receptors. We investigated the effects of 2'-fucosyllactose (2'FL), 6'-sialyllactose (6'SL), 3'-sialyllactose (3'SL) and lacto-N-neoTetraose (LNnT) on human respiratory epithelial cell lines or peripheral blood mononuclear cells (PBMCs) following respiratory viral infection *in vitro*. Expression of cytokines and viral load were monitored in infected cells. These biomarkers of innate immunity were selected since viral load and cytokine levels (IP-10, MIP-1α, IL-6, IL-8, TNF-α) have been correlated with disease severity in respiratory syncytial virus (RSV) and influenza (IAV) virus infection *in vivo*. 2'FL significantly decreased RSV viral load and cytokines associated with disease severity (IL-6, IL-8, MIP-1α) and inflammation (TNF-α, MCP-1) in airway epithelial cells. LNnT and 6'SL significantly decreased IAV viral load in airway epithelial cells. 6'SL dose-dependently down-regulated IP-10 and TNF-α in RSV infected PBMCs. HMO at or below levels found in breast milk enhance innate immunity to respiratory viruses *in vitro* and may interact directly with cells to modulate biomarkers of innate immunity.

Keywords

Human Milk Oligosaccharides, Respiratory Syncytial Virus, Influenza Virus, Inflammation, Innate Immunity

1. Introduction

Human milk oligosaccharides (HMO) contribute to innate immunity through several potential paths. HMO sup-

How to cite this paper: Duska-McEwen, G., Senft, A.P., Ruetschilling, T.L., Barrett, E.G. and Buck, R.H. (2014) Human Milk Oligosaccharides Enhance Innate Immunity to Respiratory Syncytial Virus and Influenza *in Vitro*. *Food and Nutrition Sciences*, **5**, 1387-1398. http://dx.doi.org/10.4236/fns.2014.514151

port initial development of infant gut microbiota by cultivating colonization with beneficial bacteria. HMO enhance growth [1] [2] and adhesion [3] of probiotic bacteria to gut epithelial cells. Bifidobacteria grown in the presence of HMO stimulate increased expression of tight junction proteins in gut epithelial cells [3] *in vitro*, suggesting HMO indirectly promote gut barrier integrity. Recently, 2'FL and LNnT were shown to promote gut epithelial cell maturation and barrier function *in vitro*, suggesting specific HMO directly promote epithelial cell maturation in the small intestine [4].

Some HMO share structural homology with host cell receptors and can block pathogen binding and infection of these host cells by acting as a "receptor decoy" by binding to the pathogen. For example, α-1,2-fucosyllated HMO bind to *Campylobacter jejuni* and inhibit pathogen binding to intestinal epithelial cells [5]. Other HMO can block pathogens or toxins by binding to the target cell surface receptor, preventing the pathogen/toxin from adhering to the cell. For instance, α-1,2-fucosyllated HMO block binding of the stable toxin from enterotoxigenic *Escherichia coli* by binding to its target receptor—guanylyl cyclase C [6].

A recent review by Yang and colleagues highlighted several viral surface lectins for glycans found on the surface of human epithelial cells as well as in human milk, suggesting intake of HMO may block or prevent viral infections directly or indirectly [7]. There is evidence that HMO can protect against infection directly by mimicking viral receptors on the cell's surface to block infection [7]-[9] or by competing with a virus (e.g., human immunodeficiency virus) to bind a C-type lectin receptor (e.g., Dendritic Cell-Specific Intercellular adhesion molecule-3-grabbing non-integrin or DC-SIGN) on a target cell's surface [10].

The effects of individual HMO on early immune responses *in vitro* to respiratory viral infection in circulating immune cells or airway epithelial cells have not been studied to date. Respiratory epithelial cells become infected with respiratory syncytial virus (RSV) or influenza (IAV) upon inhalation or inoculation of the respiratory mucosa [11]. Infection then spreads along the respiratory mucosa through cell-to-cell transfer (RSV) [11] [12], viral budding (IAV) [13] and aspiration of nasopharyngeal secretions [12]. Others have shown that RSV viral replication and inflammatory processes occurring in nasal air passages reflect those in lower airways [14] [15]. Since HMO bathe the laryngopharyngeal region during oral consumption, it has been postulated that HMO may reduce pathogen adhesion at the entry of the upper respiratory tract [16]. Cells exposed to HMO in the laryngopharyngeal region would include respiratory mucosal epithelial cells as well as locally resident or transient immune cells (lymphocytes, dendritic cells, monocytes, macrophages, NK cells and M cells) in the palatine and lingual tonsils. HMO have been shown to dampen platelet-neutrophil mediated inflammation [17] [18] and have been shown to be absorbed into the peripheral circulation [19] [20] with potential access through tonsils or the gut mucosa [21] [22]. For these reasons, we investigated the effects of HMO interaction with human respiratory epithelial cells or peripheral blood mononuclear cells (PBMCs) during respiratory viral infection *in vitro*. We monitored HMO effects on virus load and cytokine expression. These biomarkers of innate immunity were selected since viral load and cytokine levels (IP-10, MIP-1α, IL-6, IL-8, TNF-α) have been correlated with disease severity in respiratory syncytial virus (RSV) [23]-[28] and influenza (IAV) [29]-[32] virus infection *in vivo*.

2. Materials and Methods

2.1. Biosynthesized Human Milk Oligosaccharides

Purity of oligosaccharides and their endotoxin levels are summarized in **Table 1**. HMO identity was confirmed by the manufacturer using nuclear magnetic resonance (NMR) to confirm chemical structure, and mass spectrometry (MS) to confirm molecular weight. HMO purity was measured by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD) using relative peak area comparisons. Moisture content was determined separately using the Karl Fischer method for moisture determination [33]. 3'-Sialyllactose (3'SL), 6'Sialyllactose (6'SL) and 2'Fucosyllactose (2'FL) were all derived from bacterial synthesis. Lacto-N-neoTetraose (LNnT) was synthesized from a yeast fermentation system and purified by crystallization [34]. The endotoxin content was measured in 10 mg HMO/mL reconstitutions using manufacturer's instructions to perform the end point chromogenic QCL-1000 Limulus Amebocyte Lysate test (Lonza). Briefly, a 50 µL sample of each reconstituted carbohydrate was mixed with an equal volume of the Limulus Amebocyte-Lysate (LAL) and incubated at 37˚C (±1˚C) for 10 minutes. Next 100 µL of substrate solution was mixed with the LAL-sample and incubated at 37˚C (±1˚C) for an additional 6 minutes. The reaction was then stopped with the addition of 100 µL stop reagent and mixed. Endotoxin present is directly proportional to the absorbance read at405 - 410 nm, so the amount of endotoxin present in each sample tested was directly calculated from the assay standard curve.

Table 1. Characteristics of carbohydrates evaluated.

Oligosaccharide	Molecular Weight (Da)	% Purity	% Moisture	Endotoxin Concentration EU/mg
3'-Sialyllactose (3'SL, acidic)	633.55	97.1%	5.0%	0.015
6'-Sialyllactose (6'SL, acidic)	633.55	96.6%	10.6%	0.496
2'-Fucosyllactose (2'FL, neutral)	488.44	95.3%	6.5%	0.375
Lacto-N-neoTetraose (LNnT, neutral)	707.63	95.2%	3.7%	0.34
Galactooligosaccharides (GOS)	342- 1314	48.4%	25%	0.58
L-Fucose	164.16	99.0%	Not Tested	Not Tested
Lactose	342.3	99.0%	0.4%	0.002
Heparan sulfate	637.5 (average)	95% - 98%	Not Tested	Not Tested

2.2. Respiratory Syncytial Virus (RSV)—A Strain

A New Mexico clinical isolate, NM232, was used at an MOI of 1. RSV NM232 was obtained from the New Mexico Department of Health and was plaque-purified three times under agarose. Following selection, one plaque was used to inoculate a subconfluent HEp-2 cell monolayer. After adsorption for 1 h at room temperature, Minimal Essential Medium with 10% low IgG fetal bovine serum was added and the infection allowed to proceed for 3 days at 37˚C until the entire monolayer showed cytopathic effects. The contents of the flask were resuspended, aliquotted, snap-frozen with alcohol/dry ice, and stored at −80˚C. Virus was derived from this master stock by infecting subconfluent HEp-2 monolayers at a multiplicity of infection (MOI) of 0.1, and harvesting the monolayer when it appeared to be completely infected. The cells and media were disrupted by repeated freeze thaw cycles and clarified by centrifugation at 1000 × g for 10 min. The supernatant was aliquotted and stored at −80˚C then thawed rapidly at 37˚C just prior to use. Viral titers were determined by plaque assay. The virus was sequenced to verify purity and genotype. No other contaminating viruses were detected and the NM232 virus was phylogenetically similar to other RSVs from the GA2 clade.

2.3. Influenza A Virus (IAV)—H1N1

A/New Caledonia/20/99 (H1N1) (NC99), a seasonal influenza strain included in the seasonal influenza vaccine for 7 years, was obtained from the Centers for Disease Control. The stock virus was passaged twice in 10-day old embryonated chicken eggs and then in Madin-Darby Canine Kidney (MDCK) cells prior to infection of lung epithelial cells described herein. Briefly, MDCK cells were infected one day post plating, when the cells were subconfluent. Prior to infection, cells were washed twice with MDCK Influenza Infection Media (Minimum Essential Medium Eagle, 25 mM HEPES, 1% L-glutamine, 1% sodium pyruvate, 0.15% sodium bicarbonate, 1X penicillin/streptomycin and 3 μg/mL TPCK-treated Trypsin). The viral solution was prepared by adding 100 μL of IAV (post passage in chicken eggs) to 24 mL of infection media, and then 12 mL of the viral solution was added to each flask of cells. Cells were incubated at 37˚C and 5% CO_2, and 2 days post infection, the MDCK cell monolayer was completely disrupted by viral infection. Flasks were scraped to remove any remaining cells, and the resulting mixture of cells and supernatant was collected, pooled and clarified by centrifugation (1000 × g for 10 min). Supernatant was collected, aliquotted and stored at −80˚C. IAV titer was determined by plaque assay. The influenza A/New Caledonia/20/99 was used at an MOI of 0.01 for infection of lung epithelial cells.

2.4. Respiratory Epithelial Cells

The 16HBE [35] epithelial cell model is a modification of Saedisomeolia and colleagues [36]. 16HBE is an upper respiratory human bronchial epithelial cell line originally derived from a 1-year-old male heart-lung transplant patient that was SV40 transformed and retains tight junctions and cilia [35]. Cells were maintained in Minimum Essential Medium with Earle's Salts, 10% Fetal Bovine Serum (FBS), 1% antibiotic and 2 mM L-glutamine. The 16HBE cells were passed and plated at 200,000 cells/well in pre-coated 12 well plates (Techno Plastic Products). The plates were pre-coated with Laboratory of Human Carcinogenesis (LHC) basal medium containing 100 μg bovine serum albumin (BSA)/mL (Sigma A2934), 27 μg collagen/mL (BD Biosciences 354,231) and 10 μg human fibronectin/mL. When the 16HBEs were approximately 80% confluent, the 24 hour pretreatment of cells with HMO and controls was initiated. See **Figure 1** for a flow diagram of the test protocol. At minimum, n = 3 for each HMO or control tested.

The Calu-3 (ATCC Catalog Number HTB-55) epithelial cell model is a modification of Harcourt and collea-

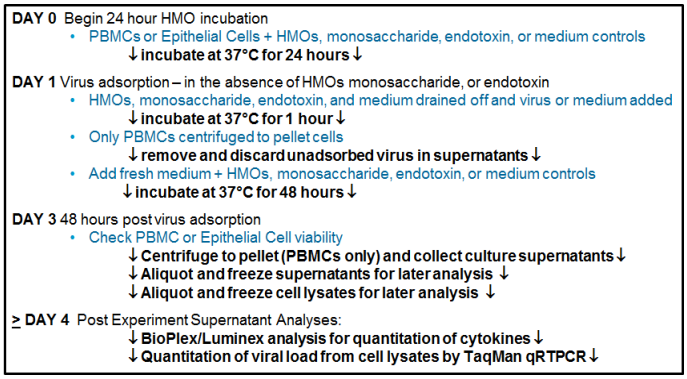

Figure 1. Test protocol flow diagram. ↓ = Upon completion move to the next step.

gues [37]. Calu-3 is a human sub-bronchial gland cell line originally derived from the lungs of a 25-year-old male patient with upper respiratory adenocarcinoma (ATCC catalog number HTB-55). Cells were maintained in Dulbecco's Modified Eagle's Medium (with 10% fetal bovine serum, 1% antibiotic and 2 mM L-glutamine). These cells were seeded at 500,000 cells per well on polyester trans-well inserts (0.33 cm, 3 µm pore, Corning #3462) and allowed to polarize. Cells were incubated for 7 days with media changes every two days. On the 7th day, the trans-epithelial electrical resistance (TEER) measured >800 Ohm (indicating the presence of tight junctions). The 24-hour pretreatment of cells with HMOs and controls was initiated for Calu-3s on Day 7. See **Figure 1** for a flow diagram of the test protocol. At minimum, n = 3 for each HMO or control tested.

2.5. Peripheral Blood Mononuclear Cells

The human peripheral blood mononuclear cell (PBMC) model is a modification of the work of Douville and colleagues [38]. The study protocol to obtain adult human PBMCs was reviewed and approved by Schulman Associates Institutional Review Board (Cincinnati, OH). Written informed consent was obtained from 5 normal healthy adults, 18 to 55 years old. PBMCs were separated from freshly drawn whole blood using Histopaque 1077 following the manufacturer's instructions. Mononuclear fractions were washed 3 times with Dulbecco's PBS then resuspended in 2 - 10 mL of PBMC culture medium (RPMI-1640 with HEPES, 1% L-glutamine, 5% FBS, 1X MEM Non-Essential Amino Acid Solution, 1X MEM Vitamins Solution, 1% Sodium Pyruvate). PBMCs were manually counted with a hemocytometer via light microscopy using equal parts of 0.4% Trypan Blue stain and PBMCS from a single donor. PBMCS were diluted with PBMC culture medium to achieve a concentration ranging from 1.6 to 1.8 × 10^6 cells/mL. Cells from a single donor were divided into snap cap tubes, with each tube containing 1 mL, and representing a single variable or control. Test articles were added to the experimental tubes, with the negative and RSV controls receiving an equivalent volume of medium alone. The 24-hour pretreatment of PBMCs with HMO and controls was initiated the same day the blood was drawn. See **Figure 1** for a flow diagram of the test protocol. At minimum, n = 4 different individuals for each HMO or control tested.

2.6. RT-qPCR

Viral gene copy number was measured as a surrogate for viral load in cell lysates from the 48 hours post-infection samples by TaqManqRT-PCR, for NS1 (RSV) [39]-[41] or M gene (IAV) expression. Briefly, cells from culture plates were placed in 350 µL TRK buffer and the RNA isolated using a QiagenRNeasy kit according to the manufacturer's instructions. 100 - 500 µg of RNA was used for the subsequent RT-qPCR assay of either NS1 (RSV) or M gene (IAV). PCR reactions utilized the Quanti Tect® Virus detection kit from Qiagen (cat. # 211011) which is a one-step real time PCR (including reverse transcription) kit specifically designed to amplify low replicating viruses. PCR reaction conditions followed the recommendations from Qiagen based on using the Applied Biosciences RT-qPCR machine (model 7900 HT). The two-step reaction was as follows: 20 min at 50˚C (reverse transcription) and 5 min at 95˚C (inactivation of reverse transcriptase), 15 s at 95˚C, 45 s at 60˚C (repeat 40 cycles).

Positive control mRNA of the NS1 gene from RSV generated from in vitro transcription was used to generate a standard curve based on copy number, allowing for quantification of the unknowns. RSV: Forward Primer se-

quence: 5'-GAG ATG GGC AGC AAT TCA TTG AGT A-3' (synthesized by IDT); Reverse Primer sequence: 5'-CAT GCA CAA ACA CAA TGC CAT CA-3' (synthesized by IDT); Probe: 5'-/56-FAM/ACG CTT TGG CTA AGG CAG TGA TAC A/36-TAMSp/-3' (synthesized by IDT). Universal influenza A primers-probes were purchased form Bioresearch Technologies (Cat # infA-p-5; InfA-R-20; InfA-F-20). Viral gene copy number was normalized to cellular 18 s RNA (Applied Biosystems; product # 4318839) copy number using multiplex TaqManRT-qPCR.

2.7. Cytokine/Chemokine Panel

The panel of cytokines and chemokines was chosen based on well described mediators of inflammation driven by respiratory viral infection. Cytokines tested include key mediators observed in severe childhood RSV infection [26]-[28] as well as severe childhood IAV infection [32]. Cell supernatants were stored frozen at −80˚C until the day they were tested for IFN-α, IFN-γ, IL-1α, IL-1β, IL-1ra, TNF-α, IL-6, IL-8, IL-10, IP-10, MIP-1α, MIP-1β, MCP-1 and RANTES following manufacturer's instructions and using a Bio-Plex/Luminex Suspension Array.

2.8. Statistics

Data were analyzed using Prism Version 5 software (GraphPad Software, La Jolla California). Differences between independent variables and the virus control were computed using a one-way ANOVA and Dunnett's Multiple Comparison Test, with the overall significance level set to $p < 0.05$.

3. Results

Weichert *et al.* [42] recently showed that 24-hour pretreatment of cells with individual biosynthesized neutral HMO (10 mg/mL) inhibit bacterial adhesion to intestinal and respiratory epithelial cell lines *in vitro* similar to pooled breast milk-derived HMO. We chose to study dose dependent effects of individual biosynthesized neutral and acidic HMO on innate immune responses to respiratory viruses *in vitro*.

3.1. Epithelial Cell Results—Viral Load

Viral load was measured to determine if HMO treatment could modulate innate immunity to respiratory viruses *in vitro*. In the 16HBE cells, the heparan sulfate positive control (**Figure 2(E)**) demonstrated a clear dose-dependent inhibition of RSV replication [43], indicating the assay system performed as expected (pre-treatment with heparan sulfate blocks RSV infection). 2'FL showed a trend toward decreased RSV viral load in 16HBE cells at concentrations as low as 5 μg/mL with the decrease reaching statistical significance at concentrations greater than or equal to 50 μg/mL (**Figure 2(A)**). In contrast, 3'SL significantly decreased RSV NS1 expression at concentrations ranging from 5 - 50 μg/mL, but not at concentrations greater than or equal to 100 μg/mL (**Figure 2(B)**). LPS, 6'-SL, GOS, fucose, and lactose (**Figure 2(A)**, **Figure 2(B)**, **Figure 2(D)**, **Figure 2(F)**, **Figure 2(G)** and **Figure 2(H)**) did not significantly decrease RSV viral load.

LNnT did not significantly decrease RSV NS1 expression in 16HBE cells (**Figure 2(C)**), but demonstrated a dose-dependent, significant decrease in IAV M gene expression at concentrations greater than or equal to 1 μg/mL (**Figure 3(A)**).

6'SL displayed a dose-dependent down-regulation of IAV M gene expression (**Figure 3(B)**), but 2'FL, 3'SL (**Figure 3(C)** and **Figure 3(D)**), LPS and lactose (data not shown) did not significantly decrease influenza viral load in 16HBE cells.

Experiments in polarized Calu-3 cells, showed some slightly different dose effects. 2'FL significantly decreased RSV NS1 expression in this upper respiratory epithelial cell line at concentrations at or above 1 μg/mL (**Figure 4(A)**). 6'SL dose-dependently decreased influenza M gene expression in Calu-3 cells, reaching significance at a slightly higher concentration of 1 mg/mL (**Figure 4(B)**). Together, these findings suggest that HMO limit *in vitro* infection of airway epithelial cells by common respiratory viruses.

3.2. Epithelial Cell Results—Cytokines

The ability of HMO treatment to reduce cytokines associated with respiratory viral inflammation was evaluated. 6'SL dose-dependently decreased IL-6 in IAV infected Calu-3 cells (**Figure 4(C)**). In 16HBE cells, 2'FL significantly decreased RSV-dependent increases in IL-6, IL-8, MIP-1α, MCP-1 and TNF-α (**Figure 5**).

3'SL significantly decreased RSV-dependent increases in IL-6, MIP-1α and IL-8 (**Figures 6(A)-(C)**) in the

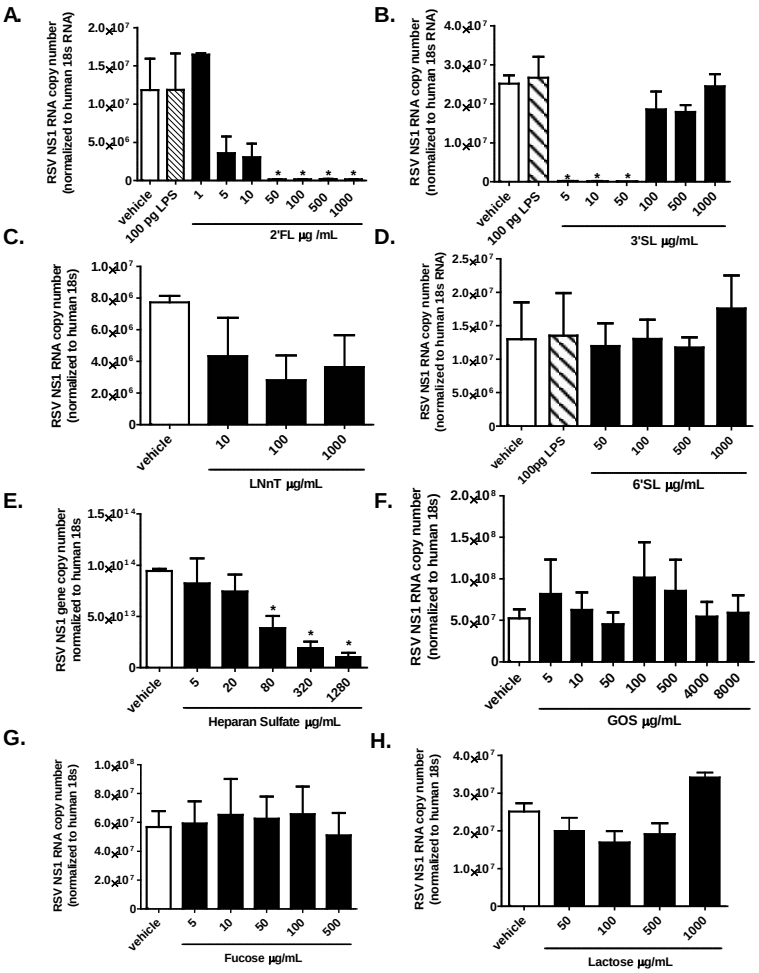

Figure 2. Differential test article effects on RSV NS1 gene expression in 16HBE epithelial cells by qRT-PCR. (A) 2'FL; (B) 3'SL; (C) LNnT; (D) 6'SL; (E) Heparan sulfate; (F) GOS; (G) Fucose; (H) Lactose. $^{*}p < 0.05$.

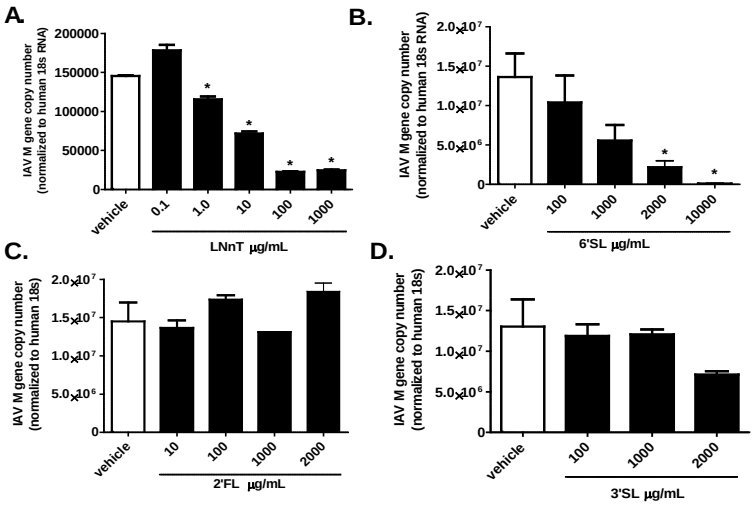

Figure 3. Differential HMO effects on IAV M gene expression in 16HBE epithelial cells by qRT-PCR. $^{*}p < 0.05$, (A) LNnT; (B) 6'SL; (C) 2'FL; (D) 3'SL.

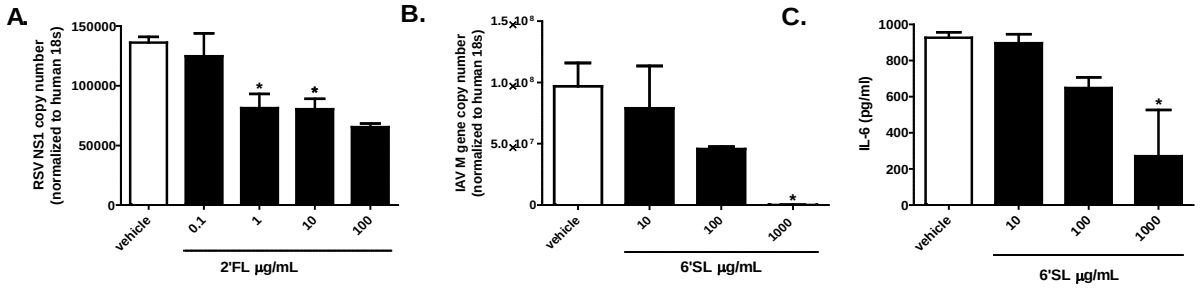

Figure 4. HMO effects on Calu-3 epithelial cells. *p < 0.05, (A) 2'FL decreases RSV NS1 expression by qRT-PCR; (B) 6'SL dose-dependently decreases IAV M gene expression by qRT-PCR; (C) 6'SL dose-dependently decreases IL-6 expression in RSV infected Calu-3 cells.

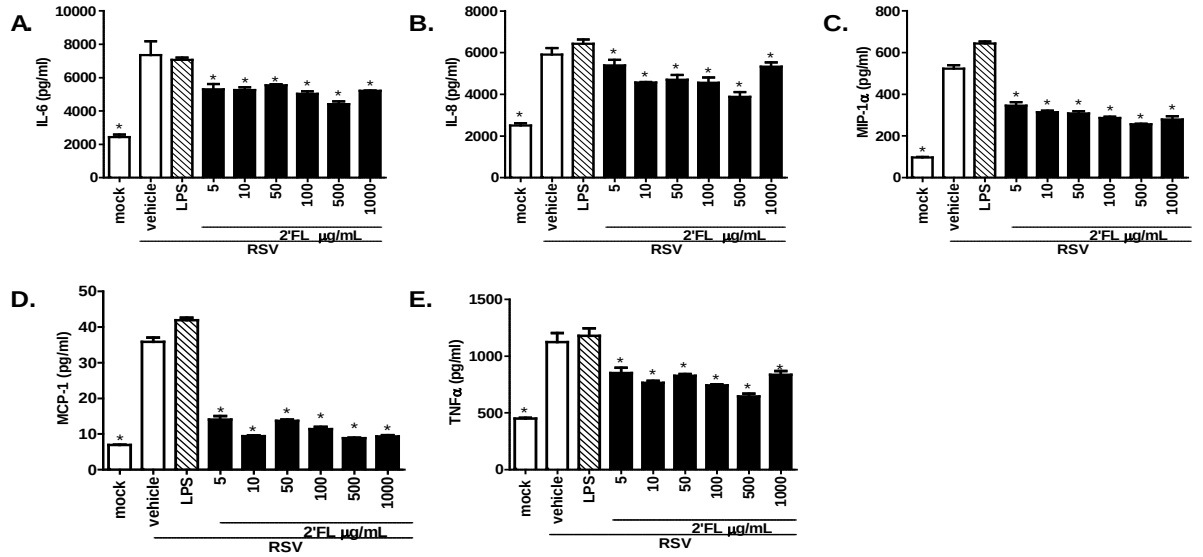

Figure 5. 2'FL decreases cytokine markers of inflammation in RSV-infected 16HBE epithelial cells. The mock control is the uninfected cell control while the vehicle control is the infected cell control. *p < 0.05, (A) IL-6; (B) IL-8; (C) MIP-1*α*; (D) MCP-1; (E) TNF-*α*.

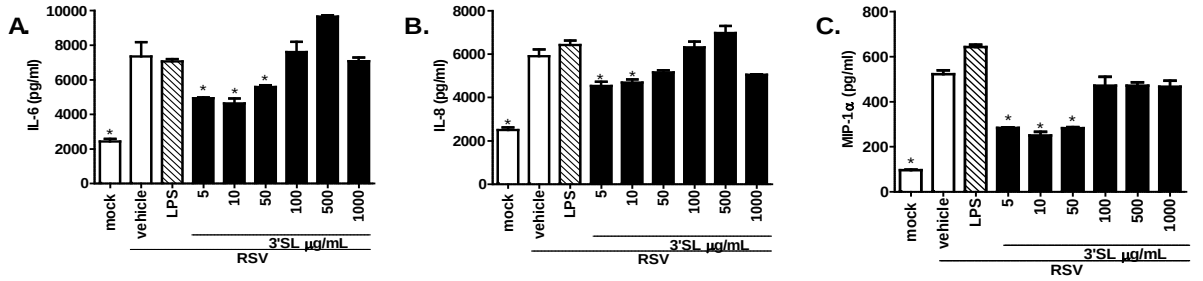

Figure 6. 3'SL decreases cytokine markers of inflammation in RSV-infected 16HBE epithelial cells. *p < 0.05, (A) IL-6; (B) IL-8; (C) MIP-1*α*. Cytokine levels mirror differences seen in RSV viral load. Lower levels of 3'SL more effective than higher levels of 3'SL in dampening inflammation and viral load.

same concentration range that significantly decreased RSV NS1 expression (**Figure 2(B)**).

3.3. PBMC Results

Lung immune cells—resident and recruited—are essential for the clearance of respiratory pathogens and their response is modulated by factors in the extracellular milieu. Adult PBMCs were used to investigate the effects of HMO and monosaccharides on PBMC response to RSV challenge. Domurat and colleagues demonstrated that

RSV will infect and replicate in human mononuclear cells, and can be found in circulating monocytes of infants with RSV infection [44]. In our experiments, RSV NS1 expression was lower in PBMCs than in epithelial cells following viral challenge and none of the HMO tested showed a significant decrease in PBMC RSV viral load (data not shown).

HMO treatment did influence PBMC innate cytokine responses to RSV challenge. 6'SL dose-dependently decreased the level of interferon-gamma inducible protein 10 (IP-10 or CXCL10) and TNF-α in RSV challenged PBMCs (**Figure 7(A)** and **Figure 7(B)**).

In addition to the effect HMO have on PBMC response to viral challenge, we observed an HMO driven alteration of innate cytokine production in unchallenged PBMCs. In the treated but uninfected PBMC, we observed a concentration-dependent increase in IL-6 as dose of 6'SL increased (**Figure 7(C)**). Together, these results indicate that HMO influence the basal and viral innate immune response of PBMCs.

4. Discussion

Our work (summarized in **Table 2**) suggests that specific human milk oligosaccharides enhance innate immunity against respiratory viruses by reducing respiratory viral infection and/or inflammation in human airway epithelial and peripheral blood mononuclear cells *in vitro*. These positive immune effects occurred at and below the physiologic concentrations of HMO found in human breast milk [45] [46]. For instance, in airway epithelia, 2'FL and 3'SL significantly decreased RSV viral load and cytokines associated with disease severity (IL-6, IL-8, MIP-1α) and inflammation (TNF-α, MCP-1) at HMO concentrations at or below 50 μg/mL. LNnT and 6'SL significantly decreased IAV viral load in airway epithelial cells. 6'SL dose-dependently down-regulated IP-10 and TNF-α in RSV infected adult PBMCs.

In our experiments, cells were incubated with HMO prior to infection, so it is possible that exposure to HMO changed the cells by activating or down regulating a pathway that made the cells more resistant to infection. The recent work of Lin *et al.* [47] showed that pretreatment of bladder epithelial cells with HMO significantly reduced internalization of uropathogenic *E. coli*. They determined that HMO pretreatment preserved epithelial

Table 2. Summary of significant HMO effects on respiratory viral infection in human cells *in vitro*. Blank area indicates no significant effects. ↓ = significant decrease.

Epithelial Cells	Marker	RSV	Influenza
2'FL	Viral Load	↓	
	Cytokines	↓IL-6, IL-8, MIP-1α, MCP-1, TNF-α	
3'SL	Viral load	↓	
	Cytokines	↓IL-6, IL-8, MIP-1α	
6'SL	Viral Load		↓
	Cytokines		↓IL-6
LNnT	Viral Load		↓
	Cytokines		To be determined
PBMCs	**Marker**	**RSV**	
6'SL	Viral Load		
	Cytokines	↓IP-10, TNF-α	

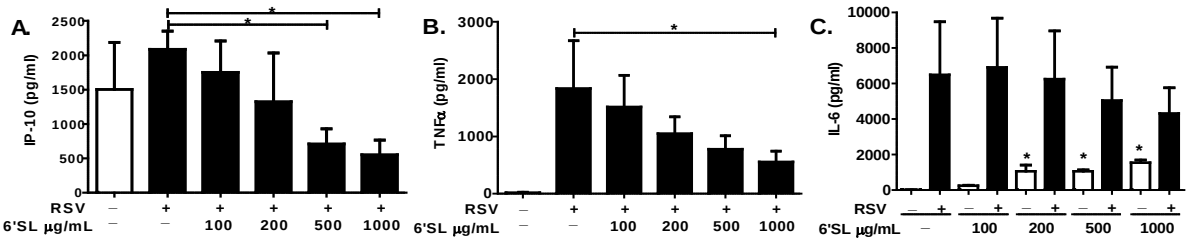

Figure 7. 6'SL decreases cytokine markers of infection and inflammation in RSV-infected PBMC. *$p < 0.05$, (A) IP-10; (B) TNF-α; (C) IL-6 (Trends). White bars show non-infected PBMC cytokine response while the black bars represent RSV infected PBMC results.

cell integrity and cell-to cell adhesion. Their work also showed that HMO, especially sialylated HMO, reduced pathogen mediated MAPK and NF-κb activation, and protected the cells from the cytotoxic and pro-inflammatory effects of infection. Understanding how HMO pretreatment impacted the cells with respect to respiratory viral infection is an area for future exploration.

Others have shown that lactadherin [48], 3'SL and 6'SL [9] dose-dependently inhibit rotavirus infection *in vitro* [9] [48] and *in vivo* [9]. HMO that inhibit norovirus binding to host cells differ by norovirus strain [8]. Pooled HMO derived from breast milk reduce binding of the HIV envelope glycoprotein HIV-1-gp120 to DC-SIGN [10]. Our data contribute additional evidence and insight into the evolving understanding of the relationship between HMO structure and function with respect to virus. Each HMO tested showed unique effects that were dependent on the type of cell involved and specific to the virus tested. The unique effects seen are likely a result of differences in expression of surface glycans and lectins between viruses tested as well as among the epithelial and immune cells utilized. Our data showed 2'FL significantly decreased RSV viral load in airway epithelial cell lines but did not decrease RSV viral load in adult PBMCs. RSV primarily infects and replicates in airway epithelium [49] but lower levels of infection and replication have also been documented in human monocytes [50], macrophages [51] and dendritic cells [52]. 6'SL dose-dependently decreased IP-10 and TNF-α in RSV-infected PBMC, but did not in airway epithelia. 6'SL significantly decreased IAV viral load but not RSV in airway epithelia. Our data also show that individual HMO effects on respiratory viral infection cannot be reproduced by individual HMO components like fucose and lactose or by a prebiotic like GOS.

Our data show dose-dependent effects on viral load (except 3'SL and RSV in epithelia), while the data for 2'FL and 3'SL (but not 6'SL) show dampening of cytokines in a non-linear way. In these instances, once a certain HMO concentration is reached, the dampening effect plateaus. This suggests that although the HMO are dampening the innate immune response, it is still intact and functioning. The dampening of the innate immune response may be a result of a decrease in viral replication and expression, or due to a direct interaction with cellular receptors that initiate or inhibit cellular pathways. The patterns and cytokines impacted by each HMO provide insight regarding mechanism of action. For example, both IFN-γ and TNF-α induce production of IP-10 in monocytes [53]. Since our PBMC data show 6'SL decreased both IP-10 and TNF-α, but did not change IFN-γ, we hypothesize that 6'SL is impacting IP-10 through TNF-α. Further research is necessary to corroborate our hypothesis.

HMO are known to act as pathogen receptor decoys [7] [54]. Since nucleolin [55] and heparan sulfate [43] are known receptors for RSV, it is possible that one or more of the HMO may block RSV binding to one of these cell surface receptors, or block fusion to inhibit viral entry. Further research is required to prove this hypothesis. Using a human milk glycan microarray, Yu [56] and colleagues have recently shown that influenza binds several α-2,6 sialylated glycans. This implies that receptor decoy is one of the potential mechanisms of action for 6'SL and influenza. However, LNnT, which is not sialylated, significantly inhibited IAV gene expression at concentrations 20 to 200 fold lower than 6'SL (**Figure 3**). Future work will focus on understanding the mechanism of action for each of these HMO to clarify their roles in the context of innate immunity.

Our results provide evidence that human milk oligosaccharides enhance innate antiviral immune responses *in vitro*. HMO that are systemically absorbed may interact directly with airway epithelial and immune cells to induce cytokines, and inhibit respiratory viral infection. HMO may also act by reducing pathogen adhesion or spread at the entry of the upper respiratory tract. Our *in vitro* studies are supportive of the epidemiological evidence that breast fed infants have fewer respiratory infections [57]-[60], but *in vivo* human clinical studies are needed to confirm these *in vitro* results.

Acknowledgements

This research was funded by Abbott Nutrition. The authors would like to thank Dr. Landon Westfall, for his contribution to this work through the influenza virus experiments. Thanks go to Dr. Dustin Starkey and Robert Ragan, for determination of quantity and purity of HMO. We are also grateful to Melissa Nameth for quantitating inherent endotoxin in the ingredients, and to Dr. Pedro Prieto for insightful review and editing of this manuscript.

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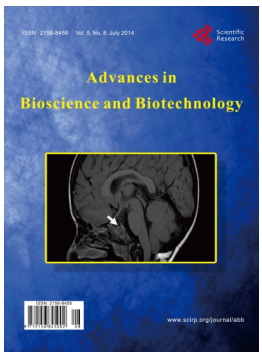

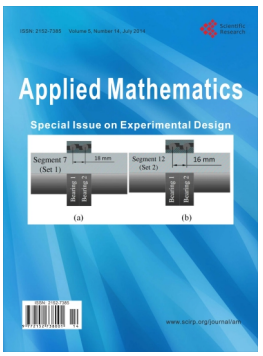

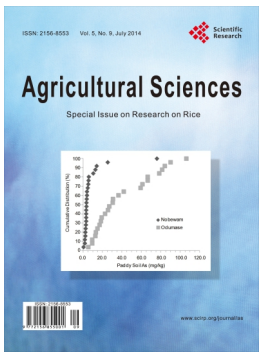

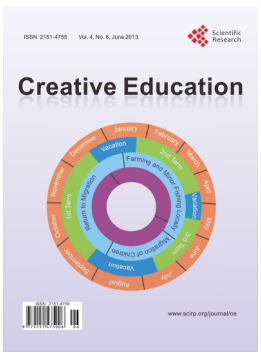

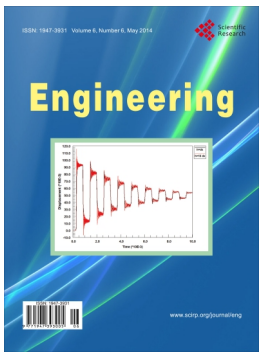

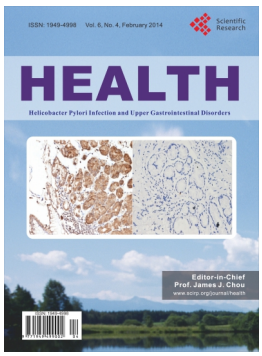

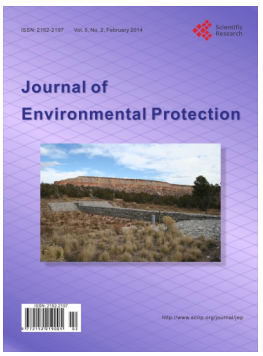

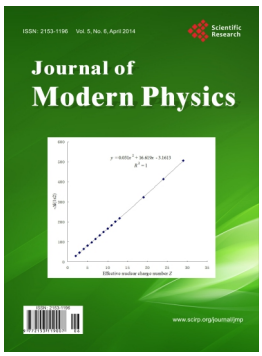

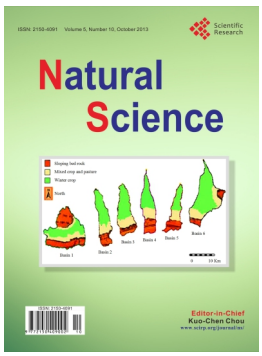

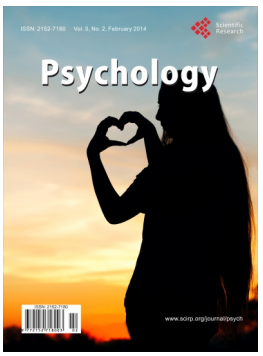

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