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The Therapeutic Potential of Human Milk Oligosaccharides in the Context of Chronic Inflammation

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Abstract

Human milk oligosaccharides (HMO) are complex unconjugated glycans that are unique to human milk and are known to benefit infant health. Preliminary data suggest that pooled HMO reduce the expression of pro-inflammatory cytokines in macrophages. Macrophages are major mediators of inflammatory pathways that contribute to chronic low-grade inflammation in many tissues and play a central role in the pathogenesis of chronic inflammatory diseases like rheumatoid arthritis (RA). The objectives of this study were (i) to identify a specific HMO responsible for the reported anti-inflammatory effects, (ii) to define the underlying molecular mechanisms using macrophage models and (iii) to assess whether or not the observed effects translate to *in vivo* efficacy in a mouse model of RA. First, RAW 264.7 macrophages were treated with the TLR4 agonist LPS to induce low-grade inflammation and co-incubation with several individual HMO revealed 3'sialyllactose (3'SL) to be most effective in decreasing *IL-1β* and *IL-6* expression. Similar findings in murine bone marrow derived macrophages (BMDM) were also confirmed and cytokine reduction was shown to be dose dependent. 3'SL also attenuated other markers of inflammation measured by both mRNA and protein secretion in BMDM. Experiments were then repeated in human macrophage THP-1 cells and similar results suggested that the effects of 3'SL were not mouse-specific. Second, the molecular mechanism behind 3'SL-attenuated inflammation in BMDM was determined by transcriptome analysis. RNA sequencing revealed that 3'SL decreased genes involved in inflammatory processes and increased lipid synthesis genes in LPS-stimulated BMDM. The identified upstream regulators of differentially expressed genes suggested that EP4 was a candidate receptor. This was confirmed when 3'SL did not attenuate *IL-6* expression in EP4-silenced RAW 264.7 macrophages. Third, the anti-inflammatory characteristics of HMO were investigated in an *in vivo* murine model of RA. Mice receiving 3'SL orally thrice daily had significantly lower ankle thickness measurements compared to control. In addition, clinical scores of severity and incidence of inflammation were significantly reduced by 3'SL treatment over time. Histology scores for inflammation, bone and cartilage erosion all showed significant reductions by 3'SL at the end of the study. These results provide first evidence of the use of 3'SL as a natural compound to target chronic inflammatory disorders. Technological advances in large-scale HMO production have increased the commercial availability and viability of 3'SL. In addition, recent safety approval of certain HMO for human consumption has paved the way for 3'SL to rapidly overcome regulatory hurdles. These developments hold promise for the future use of 3'SL in clinical research studies and its commercial application for the treatment of RA and other chronic inflammatory disorders in both children and adults.

Abbreviations

°C	degree Celsius
~	approximately
%	percentage
µg	microgram
µl	microliter
µM	micro molar
2AB	2-aminobenzamide
2’FL	2’fucosyl-lactose
3FL	3fucosyl-lactose
3’SL	3’sialyl-lactose
6’SL	6’sialyl-lactose
AA	arachidonic acid
Ab	antibody
agLDL	aggregated low density lipoprotein
aHMO	acidic human milk oligosaccharides
AP-1	activator-protein 1
APC	antigen presenting cells
ATCC	american type culture collection
BAI-1	brain angiogenesis inhibitor-1
BCA	bicinchoninic acid
BF	breast-fed
BMDM	bone marrow derived macrophages
CAIA	Collagen antibody induced arthritis
CCL	chemokine (C-C motif) ligand
cDNA	complementary deoxyribonucleic acid
CE	cholesteryl esters
CLP	common lymphoid progenitor
cm	centimeter
CMP	common myeloid progenitor
COX	cyclooxygenase
CRD	carbohydrate recognition domains
Ct	cycle threshold
C-terminus	carboxy-terminus
CVD	cardiovascular diseases
DAVID	database for annotation visualization and integrated discovery
DC	dendritic cells
DFLNH	difucosyl-lacto-N-hexaose

DFLNT	difucosyl-lacto-N-tetraose
DHA	docosahexanoic acid
DMARD	disease-modifying anti-rheumatic drugs
DMSO	dimethylsulfoxide
DMEM	dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DoHAD	developmental origins hypothesis for health and disease
DSL	disialyllactose
DSLNT	disialyllacto-N-tetraose
ECL	electrochemiluminescent
ECM	extracellular matrix components
EDTA	etylenediaminetetraacetic acid
e.g.	example
ELISA	enzyme-Linked Immunosorbent Assay
EP4	prostaglandin E_2 receptor 4
EPRAP	prostaglandin E_2 receptor 4 receptor-associated protein
ERK1/2	extracellular signal-regulated kinase1/2
EtOH	ethanol
FBS	fetal bovine serum
FDR	false discovery rate
FET	fisher's exact test
FF	formula fed
FLNH	fucosyl-lacto-N-hexaose
FPLC	Fast-protein liquid chromatography
Fuc	L-fucose
FUT2	α1-2-fucosyltransferase
FUT3	α1-4-fucosyltransferase
g	gram
Gal	D-galactose
GIT	gastro-intestinal tract
Glc	D-glucose
GlcNac	N-acetylglucosamine
GlyCAM-1	Glycosylation dependent Cell Adhesion Molecule
GM-CSF	granulocyte macrophage colony-stimulating factor
GMP	granulocyte–macrophage progenitor
GPCR	G protein-coupled receptor
h	hour(s)
H&E	hematoxylin and eosin
HIV	human immunodeficiency virus

HMO	human milk oligosaccharides
HOMER	hypergeometric optimization of motif enrichment
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HSC	hematopoietic stem cells
HEU	HIV-exposed, uninfected
IC_{50}	the half maximal inhibitory concentration
Ig	immunoglobulin
i.g.	intragastric
IKK	inhibitor of κB kinase
IL	interleukin
INF	interferon
iNOS	nitric oxide synthase
i.p.	intraperitoneal
IPA	ingenuity pathway analysis
IQ	intelligent quotient
IRAK	*IL*-1R-associated kinases
IRF3	interferon regulatory factor 3
i.v.	intravenous
JAK-STAT	janus kinase-signal transducer and activator of transcription
JNK	c-Jun NH_2-terminal kinase
JRA	juvenile rheumatoid arthritis
KO	knockout
L	liter
LBP	LPS binding protein
LCPUFA	long chain polyunsaturated fatty acids
LDL	low-density-lipoprotein
Le	lewis gene
LOX	lipoxygenase
LNFP	lacto-N-fucopentaose
LNnT	lacto-N-neo-tetraose
LNT	lacto-N-tetraose
LNFP	lacto-N-fucopentaose
LNDFH	lacto-N-difuco-hexaose
logFC	log-fold change
LPS	lipopolysaccharides
LST	sialyl-lacto-N-tetraose
M	molar (mol/L)
M1	classically activate macrophages

M2	alternatively activated macrophages
MAPK	mitogen-activated protein kinase
MCSF	macrophage colony-stimulating factor
MEK	mitogen-activated protein kinase kinase
mg	milligram
min	minute(s)
mL	milliliter
mM	millimolar
mRNA	messenger deoxyribonucleic acid
MyD88	myeloid differentiation primary response gene 88
n	number of samples
NEC	necrotizing enterocolitis
Neu5Ac	N-acetylneuraminic acid
NF-κB	nuclear transcription factor kappa-B
nHMO	neutral human milk oligosaccharides
NK	natural killer
nm	nanometer
N-terminus	amino-terminus
oxLDL	oxidized low density lipoprotein
PBS	phosphate-buffered saline
PBMC	peripheral blood mononuclear cells
PFA	paraformaldehyde
PGE_2	prostaglandin E_2
pH	negative logarithm of the molarity of H+
pHMO	pooled human milk oligosaccharides
PMA	phorbol 12-myristate 13-acetate
PNC	platelet-neutrophil complex
PRR	pattern recognition receptor
PTGER4	gene encoding prostaglandin E_2 receptor 4
QAE	quaternary aminoethyl
qRT-PCR	quantitative real-time polymerase chain reaction
q-PCR	Quantitative polymerase chain reaction
RA	rheumatoid arthritis
rcf	relative centrifugal force
RNA	ribonucleic acid
ROS	reactive oxygen species
RPM	rounds per minute
sBMO	sialylated bovine milk oligosaccharides
SDS	sodiumdodecylsulphate

Se	secretor gene
SEM	standard error of mean
Seq	sequencing
Sia	sialic acid
siRNA	short interfering ribonucleic acid
sLeX	sialyl-Lewis X
SLN	sialyllactosamine
TAK1	transforming growth factor-β-activated kinase 1
TBP	TATA-binding protein gene
TGF-β	transforming growth-factor 1
TIR	Toll-*IL*-1 receptor
TIRAP	toll-interleukin-1-receptor domain containing adaptor protein
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factor alpha
TOFMS	time of flight mass spectrometry
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing INF-β
U	unit(s)
UCSD	University of California San Diego
USA	United States of America
WBC	white blood cells
WT	wild-type

1 Introduction

1.1 Human milk – the gold standard for infant nutrition

Milk is the sole source of nutrition for the offspring of all mammalian species and for many centuries human milk has been considered the gold standard for infant nutrition (reviewed in Radbill, 1981). With evolution, human milk has become species-specific and has adapted to supply the developing infant with its biological requirements for optimal growth and survival. Several decades ago the "fetal origins hypothesis" arose from the notion of perinatal programing, which associated early life nutrition with adulthood diseases (Barker et al., 1998). The developmental origins hypothesis for health and disease (DOHaD) has recently emerged with a similar concept that proposes that perinatal programming follows exposure to environmental and nutritional factors and that essential stages of prenatal and postnatal fetal development modulate permanent changes in developmental processes (Amarasekera et al., 2013; Yang, 2012). Many metabolic and immune diseases such as type II diabetes and asthma are thought to be associated with nutrition and perinatal immune programming (Amarasekera et al., 2013; Yang, 2012). It is likely that the beneficial components of milk in early infant nutrition prime the immune system to affect health outcomes later in life. This concept may also explain the major developmental and immunological differences reported between breast-fed and formula-fed infants (Ziegler, 2006).

1.1.1 Human milk constituents and variations

Human milk is the most natural and ideal source of nutrition for the infant. It has a unique composition, which includes the necessary components for infant growth and development. Human milk is made up of mostly water (88%) and contains various concentrations of macro- and micronutrients that act as bioactive factors with many beneficial properties (Ballard & Morrow, 2013). It is ever-changing, from the beginning of a feeding to the end, from feeding to feeding, and from day to day.

Micronutrients (including vitamins A, B1, B2, B6, B12, D and iodine) vary in human milk depending on maternal diet and body stores (Valentine & Wagner, 2013). For example, low maternal exposure to sunshine can drive Vitamin D concentrations to be low, whereas Vitamin K is extremely low regardless of maternal diet (Greer, 2001). Macronutrients in human milk can also vary greatly. Proteins are divided into whey and casein and include a variety of other proteins and peptides (Gao et al., 2012; Liao et al., 2011) such as α-lactalbumin, lactoferrin, secretory immunoglobulin IgA, lysozyme and serum albumin, to name a few (Lönnerdal, 2004). Reports indicate that protein content in milk of mothers who deliver preterm is significantly higher than that of term-milk. After the first 4 to 6 weeks of

lactation concentrations decrease (Bauer & Gerss, 2011) and although not affected by maternal diet, protein increases with maternal body weight and decreases with the production of higher volumes of milk (Nommsen et al., 1991).

The most variable macronutrients of milk are lipids, which have been shown in one study to be significantly lower during night and morning feedings compared to afternoon or evening feedings (Kent et al., 2006). Another study found that milk fat varies within a single feed and that milk contains two to three times the amount of fat at the end of feeding, compared to the beginning of feeding. Certain fatty acids such as long chain polyunsaturated fatty acids (LCPUFA) and docosahexanoic acid (DHA) can fluctuate in concentrations according to maternal diet (Martin et al., 2012; Valentine et al., 2010, 2013). The least variable of the macronutrients is lactose (Ballard & Morrow, 2013), which is also the most abundant component of human milk, followed by lipids and then human milk oligosaccharides (HMO), which often exceed total protein concentrations. HMO are a family structurally diverse unconjugated glycans that are unique to human milk and provide an array of benefits to the developing infant (reviewed in Bode 2012).

1.1.2 Human milk oligosaccharides

The benefits of breast-feeding were initially described in the early 19th century, when infant mortality rates rose as high as 20-30% within the first year of life. Pediatricians observed that the mortality rates in breast-fed infants were significantly lower when compared to infants fed bovine milk, pure carbohydrates, or processed foods. This alarming health outcome led to the beginning of research on chemical components and the in depth analysis of milks, in order to provide infants with an adequate substitute for human milk (Nützenadel, 2010). It wasn't until several decades later with technological innovation, that the composition of formula resembled that of human milk and thus became standardized for commercialization (Kjeldahl, 1883; Rubner, 1889; Voit, 1881). Human milk oligosaccharides (HMO) were discovered by scientists and physicians with diverse perspectives and interests, who were respectively trying to characterize the unique and abundant carbohydrates in human milk, or trying to understand the apparent health benefits associated with breast-feeding. Scientists noticed that human milk contained "a different type of lactose" than bovine milk and later realized that the lactose was the same, but that there was an additional unknown carbohydrate fraction in human milk (Montreuil, 1992). Nearly half a century later, the carbohydrate fraction was characterized as "gynolactose" (Polonowski & Lespagnol, 1929, 1931) and later divided into individual oligosaccharides by two-dimensional paper chromatography (Polonowski & Montreuil, 1954). During that same time, physicians noted that the higher survival rates in breast-fed infants were related to their resistance to diarrheal diseases, which consequentially led to the discovery of intestinal microbiota and the

importance of milk carbohydrates for the growth of these microorganisms (Escherich, 1989; Tissier, 1900). When *Bifidobacteria* was found to be unique to the feces of breast-fed infants (Moro, 1900), further research led to the identification of a “bifidus factor” in the carbohydrate fraction of human milk (Gyorgy et al.,1954; Schönfeld, 1926), now known as HMO.

1.1.3 Biosynthesis and composition of human milk oligosaccharides

By 1965, fourteen different HMO had been identified (Jahrg et al., 1962; Kuhn & Baer, 1938; Kuhn et al., 1956, 1958; Kuhn & Brossmer, 1956; Kuhn & Gauhe, 1965; Kuhn & Klesse, 1958; Montreuil, 1956) and to date the structures of more than 100 HMO have been characterized and described (Kobata, 2010; Urashima, et al., 2009; Urashima et al.,2011). In addition, the analysis of human milk by microfluidic chip high-performance liquid chromatography (HPLC)-time of flight mass spectrometry (TOFMS), allows for the possible detection of more than 200 HMO (Ninonuevo et al., 2006).

HMO are composed of various combinations of five monosaccharides: galactose (Gal), glucose (Glc), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (Sia) in the form of N-acetylneuraminic acid (Neu5Ac) (see Figure 1.2 A). They follow a common blueprint where all HMO contain lactose (Galβ1-4Glc) at their reducing end (see Figure 1.2 B) (reviewed in Bode 2012). Lactose synthesis (Figure 1.1) occurs within the secretory cells of the lactating mammary glands. Glucose (Glc) present in the cytosol is activated to UDP-Glc and converted to UDP-Gal. UDP-Gal and additional Glc are transported into the golgi apparatus, where the enzyme complex lactose synthase catalyzes the process of transgalactosylation, resulting in the production of lactose. Lactose synthase consists of two proteins (Brodbeck & Ebner, 1966), β1-4 galactosyltransferase (β4GalT1) and α-lactalbumin. β4GalT1 transfers UDP-Gal to terminal GlcNAc during glycoconjugate biosynthesis and is present in all tissues. The expression of α-lactalbumin is regulated by lactation hormones and therefore is only present in the mammary gland and milk. When β4GalT1 binds α-lactalbumin, the acceptor specificity is altered from GlcNAc to Glc to generate lactose (Ramakrishnan et al., 2002).

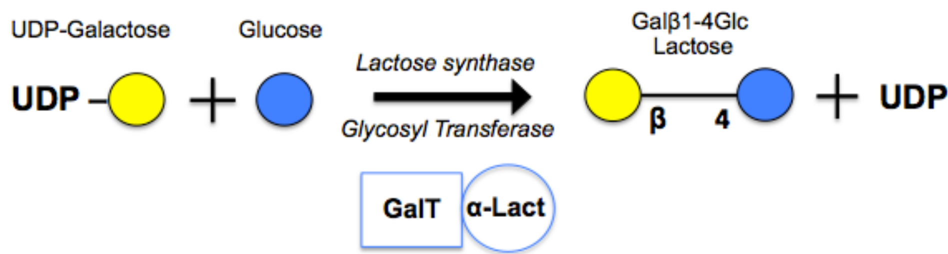


Figure 1.1 Lactose biosynthesis.
The monosaccharide galactose Gal is transferred from Uridine-diphospho galactose (UDP) to glucose (Glc) by the lactose synthase complex, galactosyltransferase and α-lactalbumin. Modified from (Castanys-Muñoz et al., 2013).

The formation of HMO from lactose is a complex process, which to this day requires additional research to provide a stronger understanding of HMO biosynthesis (Bode, 2012). HMO are thought to be produced by glycosyltransferases of epithelial cells within the mammary gland through the transfer of monosaccharide residues to lactose, or small lactose-based oligosaccharides. The lactose backbone is elongated by the disaccharide unit β1-3- or β1-6-linked lacto-N-biose (Galβ1-3GlcNAc-, type 1 chain), or N-acetyllactosamine (Galβ1-4GlcNAc-, type 2 chain) (See Figure 1.2 C). The formation of type 1 and type 2 disaccharide units is believed to result from the actions of β3GalT and β4GalT respectively (Almeida et al., 1997; Isshiki et al., 1999; Sasaki et al., 1997). When lactose is elongated with lacto-N-biose further extension of the chain ends, whereas the elongation with N-acetyllactosamine allows for the addition of a β-linked Gal to either the 3 or 4 positions of GlcNac. Linear structures without branching are known as para-HMO. The addition of a β1-6 linkage between two disaccharide units allows for branching and these branched HMO are named iso-HMO (Figure 1.2 D). β3GlcNAcT (iGnT) is thought to be responsible for linear chain elongation of HMO, while chain branching could be initiated by β6GlcNAcT (IGnT) (Ropp et al., 1991) Furthermore, the lactose backbone or the elongated oligosaccharide chain can be sialylated in α2-3 or α2-6 linkage (Figure 1.2 E) and/or fucosylated in α1-2, α1-3 or α1-4 linkage (Figure 1.2 F) and are always found in terminal positions (described in 1.1.3.1 and reviewed in Bode, 2012).

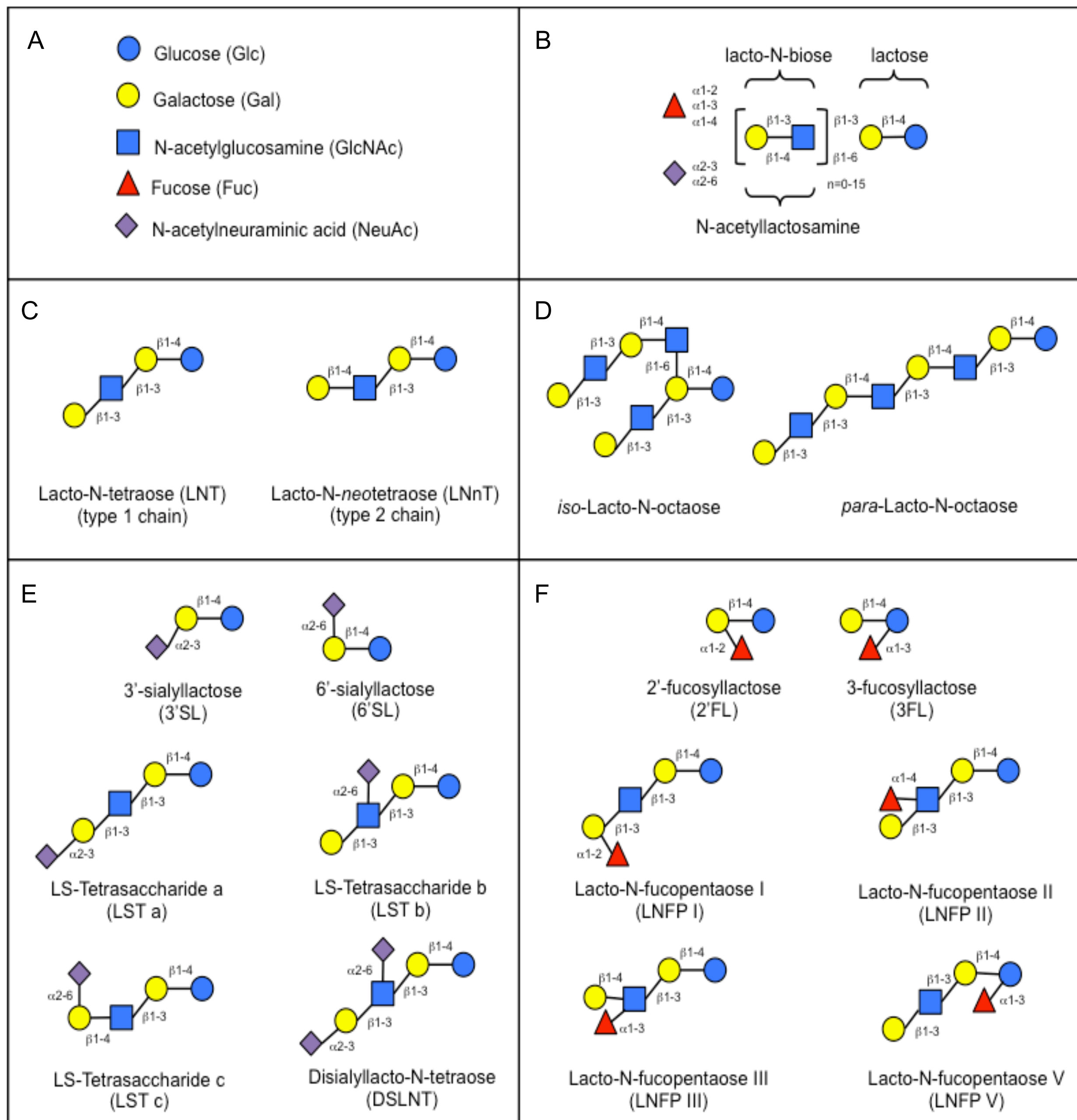


Figure 1.2 HMO blueprint and various structures.
Monosaccharides that make up HMO basic blue print (A). Structural composition of HMO (B). Lactose can be elongated by addition of either lacto-N-biose (type I) or N-acetyllactosamine (type II) disaccharides (C). β1-6 linkage between two disaccharides introduces chain branching (iso-HMO) and addition of disaccharides to each other in the β1-3 linkage leads to linear chain elongation (para-HMO) (D). Lactose or elongated type I or II can be sialylated in different linkages (E). Lactose or elongated type I or II can be fucosylated (F). Modified from Bode & Jantscher-Krenn, 2012.

1.1.3.1 Biosynthesis of fucosylated HMO

The first indication of variations of HMO amongst women's milk was in 1967, when scientists observed that 2'-fucosyllactose (2'FL) was missing in certain milk samples collected from mothers of "non-secretor" blood type (Grollman & Ginsburg, 1967). Non-secretor women do not have the secretor gene (Se) and therefore cannot produce the soluble form of H antigen in secretions from epithelial cells of mucous glands (such as milk, saliva and mucus) (Lowe, 1993). The addition of Fucose (Fuc) to oligosaccharides in human milk is regulated by several fucosyltransferases (FuT), such as the enzyme α(1,2)fucosyltransferase (FuT2), which is encoded by the Se gene (Kelly et al., 1995). Fut2 transfers α-fucose to the 2 position of the terminal Gal and thus only in the milk of secretor mothers does FuT2 use lactose as a substrate (Castanys-Muñoz et al., 2013) to produce α1-2-fucosylated oligosaccharides, such as 2'FL or lacto-N-fucopentaose I (LNFP I). The other important enzyme for the addition of Fuc is α(1,3/4)fucosyltransferase (FuT3), which is encoded by the Lewis gene (Le) (Kukowska-Latallo et al., 1990). FuT3 transfers Fuc from GDP-Fuc to GlcNac in either α(1-3) or α(1-4) linkages, and can also transfer Fuc to the 3 position of glucose to produce 3Fucosyllactose (3FL) and difucosyllactose (DF-L) (Larsen et al., 1990). Individuals with the Le gene are termed "Lewis positive" and can be either secretors or non-secretors. Non-secretors produce the Lewis a antigen (Lewis a+b-), whereas secretors produce the Lewis b antigen (Lewis a-b+) (Grollman et al., 1969). Although individual milk samples can be classified into four groups (Figure 1.3), HMO composition in milk remains rather complex. FuT2 and FuT3 compete for the same substrates, which leads to the variation of HMO profiles between women (Johnson & Watkins, 1992; Kumazaki & Yoshida, 1984; Xu et al., 1996). Research indicates that other FUT (FUT1, 4, 5, 6, 7 or 9) may also be involved in HMO fucosylation, resulting in the detection of 3FL, LNFP III or α1-2-fucosylated HMO, in the milk of women whom express neither FUT2 nor FUT3 (Newburg et al., 2005). Interestingly, five percent of women who are Lewis negative (Lewis a-b-) have milk and colostrum devoid of LNFP II, a known Fuc(α1-4) GlcNAc-containing HMO (Kobata, 2010). Reports have identified that the functional FUT2 alleles prevail by over 70% in most geographical regions of the world. In some regions of Asia and Africa non-functional FUT2 alleles were reported to be just as prevalent (Castanys-Muñoz et al., 2013). All together these findings help address the intrapersonal variations in fucosylated HMO amongst lactating women due to genetic diversity.

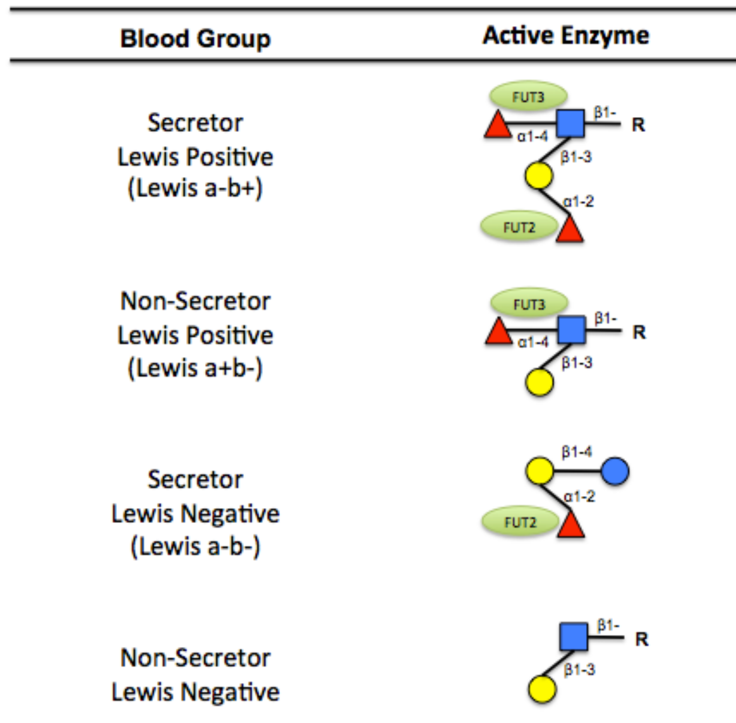


Figure 1.3 Blood groups and enzyme activity.
Fucosylation depends on the Secretor and Lewis blood group and results in four milk groups. Secretors express the gene encoding FUT2, which transfers Fuc in an α1-2 linkage to terminal Gal. The Lewis gene encodes FUT3, which adds Fuc in an α1-3 or α1-4 linkage to subterminal GlcNAc. Modified from (Bode & Jantscher-Krenn, 2012; Castanys-Muñoz et al., 2013).

1.1.3.2 Biosynthesis of sialylated HMO

The addition of sialic acids to HMO appears to be regulated by several sialyltransferases. Their expression and regulation is complex and how they contribute to interpersonal variations and the relative abundance of sialylated HMO has yet to be fully understood (Maksimovic et al., 2011). The two genes expressed in the lactating mammary gland are thought to be ST6Gal1 (responsible for the synthesis of 6'SL and LSTc) and ST3gal4 (for the synthesis of 3'SL) (Fuhrer et al., 2010). Whether one of these transferases also sialylates disialyl-LNT (DSLNT), which is α2-3-sialylated at the terminal Gal, remains unknown. The HMO LSTb has a specific structural feature, where the sub-terminal GlcNAc is sialylated in an α2-6 linkage, but the terminal gal is not (Figure 1.4). According to Tsuchida et al. (2003), this is rarely observed on human tissues with the exception of certain tumors and the central nervous system. Our knowledge of HMO sialylation remains fairly limited and questions still remain unanswered (Bode, 2012).

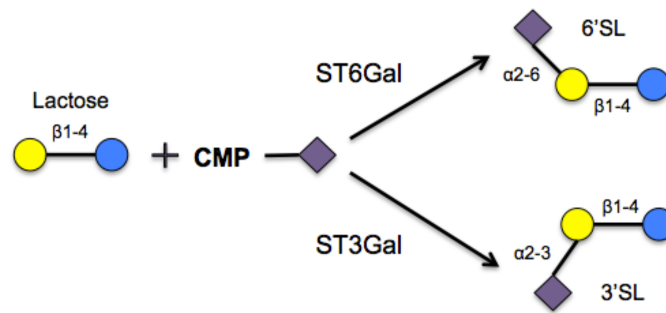


Figure 1.4 Synthesis Sialylated HMO.
Free Sia is transferred via the sugar donor citidine monophosphate-N-acetylneuraminic (CMP) to lactose, by linkage specific sialyltransferases, ST3Gal and ST6Gal, to form sialylated HMO 3′sialyllactose (3′SL) and 6′sialyllactose (6′SL), respectively. Modified from (Castanys-Muñoz et al., 2013)

1.1.3.3 Variations in HMO

Different women synthesize different subsets of oligosaccharides, resulting in a variation in total amount and relative abundance of HMO across individuals. Since secretor status is a predictor of fucosylated HMO (Erney et al., 2001) (also described in 1.1.3.1), genetic variability also drives differences in HMO composition geographically. Recently, McGuire et al. (2017) examined the relationship between HMO composition and genetic variations across populations in 11 different locations of the world. Results revealed that mean 2'FL concentrations were three to five times higher in milk collected from Hispanic populations compared to those in Ghana. Differences in LNnT and DSLNT were also found between populations of similar ethnicity, living in different locations (McGuire et al., 2017). In addition to genetic differences, environmental factors are also thought to influence HMO diversity (Erney et al., 2000; Musumeci et al., 2006; Radzanowski et al., 2013). Maternal age, time postpartum, weight, and BMI, were also reported to be associated with compositional differences in HMO (McGuire et al., 2017). An earlier study indicates that total amount of HMO is significantly higher in women with a BMI between 24 and 28 compared to women with a lower BMI between 14 and 18 (Nissan et al., 2010). It was not investigated whether specific HMO contributed to the observed differences, and how nutrition affects HMO synthesis has yet to be determined.

HMO concentrations change over the course of lactation and therefore vary greatly within a single individual (Figure 1.5). For example, colostrum which is produced by the mammary glands in late pregnancy and for the first few days after parturition, can contain concentrations of HMO up to 20-25 g/L (Coppa et al., 1999; Gabrielli et al., 2011). In mature human milk, HMO range from 5-20 g/L and have even been reported to exceed

concentrations of lactose in the milk of mothers with preterm infants. (Bode, 2012; Gabrielli et al., 2011). In addition, sialylated HMO concentrations have been reported to peak at the start of lactation and decrease overtime (Wang et al., 2001).

Categories of HMO (%total)	Oligosaccharide	Mean Concentration (range) g/L
Fucosylated (35-50%)	2'FL	2.7 (1.88-4.9)
	3FL	0.5 (0.25-0.86)
	LNFP-I	0.122 (0.106-0.145)
	LNFP II, and III	0.156 (0.120-0.161)
Sialylated (12-14%)	3'SL	0.2 (0.1-0.3)
	6'SL	0.5 (0.2-1.22)
Nonfucosylated neutral (42-55%)	LNnT	0.3 (0.17-0.45)

Figure 1.5 Reported HMO concentrations in human milk from different studies. The inter- and intrapersonal variations in HMO determine the composition and relative abundance of individual HMO in a given milk sample. (Adapted Donovan & Comstock 2016; Smilowitz et al., 2014; Kunz et al., 2016; Thurl et al., 2010; Martin-Sosa et al., 2003)

1.1.3.4 Mammalian differences in milk oligosaccharides

In addition to intra- and interpersonal differences, the abundance and complexity of milk oligosaccharides are inherently unique to humans. The diversity of HMO differs greatly across species. In primates oligosaccharide concentrations reach up to only half of what is found in human milk (Urashima et al., 2013). The composition of bovine milk, which is used as basis for infant formula, varies drastically in comparison to that of humans in terms of macronutrients and HMO concentrations, as well as HMO composition (Bao et al., 2007; Chaturvedi et al., 2001; Coppa et al., 1999; Davidson et al., 2004; Gabrielli et al., 2011; Kunz et al., 1999; Newburg et al., 2002) (Figure 1.6). More than 80 acidic (or sialylated) and neutral (non-sialylated) oligosaccharides have been isolated from human milk and their chemical structures have been established (Newburg & Neubauer, 1995). In contrast, bovine milk and colostrum are deemed simple, containing only ten sialylated oligosaccharides (Kuhn & Brossmer 1956; Schneir & Rafelson 1966; Veh et al., 1981; Parkkinen & Finne 1985; Parkkinen & Finne 1983) and eight neutral oligosaccharides (Saito et al., 1984; Saito et al., 1981). Although higher concentrations of total HMO are present in bovine colostrum, as milk production matures concentrations decline and are only found in trace amounts (Gopal & Gill, 2000). The HMO 3'SL, followed by sialyllactosamine (SLN), 6'SL, and disialyllactose (DSL), are the most abundant acidic HMO and account for more than 50% of the total oligosaccharides in bovine colostrum. In contrast, concentrations of 3'SL and 6'SL account

for approximately 10-20% of total HMO in human milk and have been reported to reach up to 1.0 g/L (McVeagh & Miller, 1997).

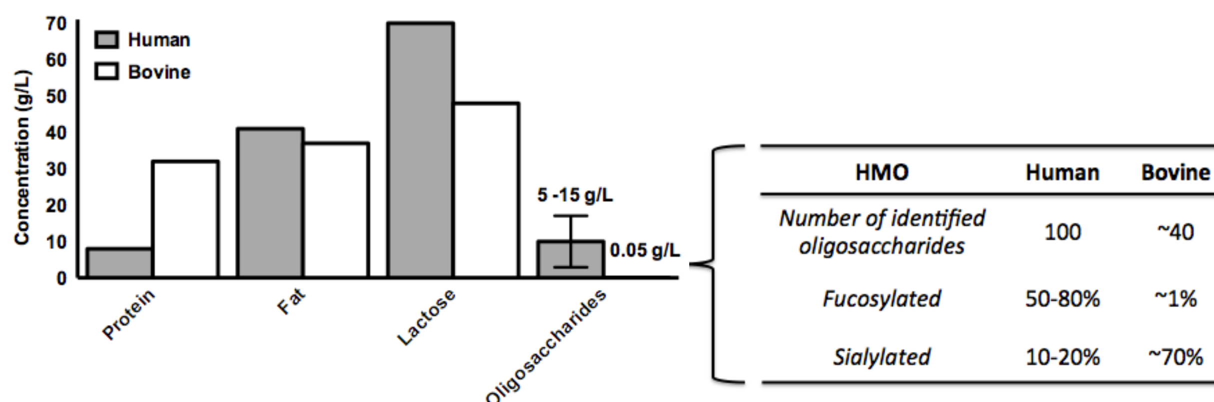


Figure 1.6 Comparisons of bovine and human milk composition.

Human and bovine milk differ in protein, lactose and oligosaccharide total concentrations. Additionally, oligosaccharide structural composition analysis from several studies, reveals fewer total sialylated and more fucosylated HMO in human milk compared to bovine milk. Modified and adapted from (Bode, 2012; Kobata, 2010; Ninonuevo et al., 2006; Tao et al., 2008, 2009, Wu et al., 2011, 2010)

1.1.3.5 Metabolism of human milk oligosaccharides

The metabolic properties of HMO were first investigated in the late 1980s and research suggested that HMO excretions in feces correlated with the secretor status and blood group of the mother, and varied with the infant's gestational age and time of fecal collection (Sabharwal et al., 1984, 1991; Sabharwal, Nilsson, Chester, et al., 1988; Sabharwal, Nilsson, Grönberg, et al., 1988). Decades later, new technology improved detection of HMO in feces and studies following infants from birth to 6 months of age revealed a gradual change in fecal HMO over time (Albrecht et al., 2010). Stable isotopes in the form of C^{13} labeled glucose or galactose (^{13}C-Gal) were administered to women as an oral bolus, providing a different method to observe the fate of ingested HMO. The studies showed that in lactating women ^{13}C-Gal was transported to the mammary gland and integrated into HMO. Within 36 hours the labeled HMO were identified in the urine of the breast-fed infants (Kunz et al., 2000; Rudloff et al., 2006, 2012). The observed HMO were unmodified and therefore validated earlier studies, where intact, unlabeled HMO were also discovered in fecal and urinary excretions (Rudloff et al., 1996). In addition, unexplained degradation products and acetylated HMO were found in both feces and urine, and lacto-N-tetraose (LNT) was present in the fecal samples. The presence of acetylated HMO was thought to be due to a modification by intestinal bacteria before absorption, or perhaps by the infant after absorption, via liver or kidney metabolism (Albrecht et al., 2011; Dotz et al., 2015;

Underwood et al., 2015). The specific stages of HMO metabolism have yet to be fully elucidated and the excretion patterns of HMO are unclear. For example, urine collected from infants fed secretor milk (predominant in LNFP-1) was completely devoid of LNFP-1. Furthermore, HMO specific to secretor status were found in the feces and urine of infants of non-secretor and Lewis negative milk (Blank et al., 2011; Dotz et al., 2014, 2015). By 6 months of age, when complimentary feeding was introduced, the presence of HMO and their metabolic by-products in feces were reduced and eventually no longer detectable as human milk feeding ceased (Albrecht et al., 2011)

HMO degradation is resilient to the low stomach pH of infants. Digestion studies using enzyme preparations of pancreas and intestinal brush border membranes indicate that HMO are not hydrolyzed by enzymes in the upper small intestine (Engfer et al., 2000; Gnoth et al., 2000). Although <5% of HMO are digested in the intestinal tract, the majority reach the large intestine and are thought to serve as prebiotics, or play a role in modulating the immune system of the infant's intestine (Gnoth et al., 2000).

More recent findings suggest that HMO are absorbed in the infant's intestine and reach the systemic circulation before being excreted in the urine and feces of breast-fed infants (Goehring et al., 2014). Although the study revealed that only 0.1% of the HMO were detectable in plasma and 1-4% in the urine, the HMO composition discovered in the infant's blood corresponded with those present in the infants' urine and their mothers' milk (Goehring et al., 2014). Cultured intestinal epithelial cells were used as a model to demonstrate that non-sialylated HMO are capable of crossing the cells' monolayers by receptor-mediated transcytosis and paracytosis, while sialylated HMO appeared to only use paracytosis (Gnoth et al., 2001). The receptors mediating HMO absorption have yet to be elucidated and the rates at which HMO are absorbed and then cleared from the system have not been determined either. A study by Rudloff & Kunz (2012) measured the intake of LNT and LNFP-1 and found that approximately 50 to 160 mg of LNT and LNFP-1 were consumed by the infants during each breast-feeding session. In addition, renal excretions were determined to reach between 1 to 3 mg of LNT and LNFP-1 per day. Altogether, these metabolic studies propose that with each day of breast-feeding hundreds of milligrams of HMO are circulating in the infant's system and are therefore expected to have effects that go beyond the gastro-intestinal tract (GIT) (Rudloff & Kunz, 2012).

1.1.4 Beneficial functions of human milk oligosaccharides

1.1.4.1 Prebiotic properties of HMO

Microbial colonization of the infant's GIT is initiated at birth. The neonatal intestine has a low microbial diversity despite the infant's exposure to vaginal and fecal microbiota during delivery (Newburg & Morelli, 2015) and microbes sampled from its local environment and surroundings. The infant's microbiota often resembles the mother's as it is also acquired from the mother's saliva, skin and milk microbiome (Bode et al., 2014; Hunt et al., 2011). Certain bacteria that make up the infant's microbiome are believed to support homeostasis and modulate inflammation and immune epithelial cell responses, thereby reducing the occurrence of inflammation and GIT infections (Cebra, 1999; Maynard et al., 2012; Nanthakumar et al., 2003; Rinne et al., 2005). Although diet has been implicated as the main driver in directing the infant's microbiome composition, the precise mechanisms by which HMO support the development of specific beneficial microbes such as *Bifidobacterium longum* subsp. *infantis* (ATCC 15697) and certain *B. breve* (e.g., SC95, SC154, SC568 and ATCC 15701) are still unclear (LoCascio et al., 2007; Ruiz-Moyano et al., 2013). Adhesion of *B. infantis* to intestinal epithelial cells is significantly improved, when cultured with HMO from pooled human milk (pHMO), or sialylated HMO 6′SL and 3′SL. Furthermore, when *B. infantis* (ATCC 15697) and *B. breve* (SC95) were first cultured with pHMO and then exposed to intestinal epithelial Caco-2 cells, a down-regulation in pro-inflammatory gene expression and an increase in the anti-inflammatory cytokine interleukin 10 (*IL-10*) was observed (Chichlowski et al., 2012; Kavanaugh et al., 2013)

Gene clusters which control the expression of glycosidases, sugar transporters and glycan-binding proteins specific to HMO utilization (Sela et al., 2008) were discovered when sequencing the genome of *B. infantis*. This may explain why *B. infantis* utilizes HMO to grow and can consume all HMO and all subsequent degradation products (Asakuma et al., 2011; LoCascio et al., 2007; Marcobal et al., 2010). Other microbes, such as *B. bifidum* (JCM1255), grow slower with HMO and leave behind unconsumed degradation products (Asakuma et al., 2011). In contrast, *B. breve* (JCM1192) and *B. longum* subsp. *longum* (JCM1217), are hardly capable of growing with HMO and only metabolize specific HMO such as LNT (LoCascio et al., 2007). Interestingly, subspecies more prevalent in adult communities such as *B. animalis* and *B. adolescentis* are unable to utilize HMO. These finding indicate that different bifidobacterial taxa implement diverse strategies in utilizing glycans, despite their common phelogenetic origins and similarities in colonizing the human GIT (Sela & Mills, 2010).

A less diverse gut bacterial composition is observed in preterm infants compared with term infants, where higher levels of Enterobacteriaceae and lower levels of Bifidobacterium and Bacteroides (Arboleya et al., 2012; Cong et al., 2016) are observed. These differences lead to intestinal dysfunction, which is one of the primary factors linked to preterm infant mortality and morbidity, and renders preterm infants highly susceptible to the development of necrotizing enterocolitis (NEC) (Nanthakumar et al., 2011) and sepsis (Torrazza & Neu, 2013). Several studies indicate that infants receiving milk lower in concentrations of DSLNT are at risk of developing NEC (Autran et al., 2017; Van Niekerk et al., 2014). In a neonatal rat model, DSLNT, but not LNT (Jantscher-Krenn, Zherebtsov, et al., 2012) reduced the incidence of NEC. Similar results were reported in the same rat model with sialylated gos - (Autran et al., 2016) and novel synthetic disialyl hexasaccharides (Yu et al., 2014), revealing that the preventative effects of HMO on NEC seem to be structure specific. Although HMO play an important role in mediating a network of relationships between the infant host and their valuable microbial community, the mechanisms are thought to go beyond a prebiotic effect, where HMO do not exclusively operate as nutrients for favorable bacteria.

1.1.4.2 Antimicrobial and antiadhesive properties of HMO

In addition to providing prebiotic functions by enriching particular bacterial taxa, HMO have been characterized to possess antiadhesive properties against infectious agents within the infant GIT (Bode, 2012; Kunz & Rudloff, 2008; Kunz et al., 2000; Morrow et al., 2005; Newburg et al., 2005). The initiation of infection begins with the attachment of pathogens to epithelial cell surfaces, which are mediated by glycan interactions. HMO isomers or subunits resembling the covalently linked cell surface glycans, can act as decoys and to inhibit pathogen attachment. The pathogen-bound HMO is then cleared through GIT motility thereby preventing the infection (Gustafsson et al., 2006; Simon et al., 1997). For example, the bacteria *Campylobacter jejuni (C. jejuni)* binds to epithelial cells via α1-2-fucosylated glycans and causes diarrhea, which in many cases leads to infant mortality. Colonization of *C. jejuni* can be reduced in mice by the administration of soluble α1-2-fucosylated HMO. In addition, binding of *C. jejuni* can be inhibited by fucosylated HMO in cultured cells and human intestinal mucosa explant models (Ruiz-Palacios et al., 2003). These findings were confirmed in a cohort study involving approximately 100 mother-infant pairs, where the incidence of *C. jejuni* diarrhea was reduced in infants fed mother's milk containing high concentrations of 2'FL. The attachment of various gastrointestinal bacteria such as *vibrio cholerae, salmonella fyris* and *enteropathogenic escherichia coli* (EPEC), was hindered by the decoy activity of non-sialylated HMO (Giovanni V. Coppa et al., 2006). Most recently, the antibacterial role of HMO against a prominent neonatal pathogen, *group B. Streptococcus* (GBS) was revealed and the bacteriostatic activity was also specific to non-sialylated HMO

and synergistic with numerous conventional antibiotic agents (Lin et al., 2017). Sialylated HMO also have antimicrobial properties and have been shown to block the adhesion of *escherichia coli* (*E. coli)* to human erythrocytes (Martín et al., 2002), indicating a structure specific mechanism, clearly mediated by HMO-receptor interactions. Studies by Angeloni et al. (2005) suggest that the 3'SL reduces the adhesion of *enteropathogenic E. coli* by modifying the surface glycan profile of Caco-2 cells and thereby changes the receptor sites for certain pathogens. This may explain why pHMO significantly reduce EPEC attachment to cultured epithelial cells and why colonization in neonatal mice supplemented with pHMO was also diminished compared to non-supplemented mice (Manthey et al., 2014). Since HMO are present in the urine of breast-fed infants, they are thought to act systemically against extra-intestinal bacterial infections. Martín-Sosa et al. (2002) confirmed these assumptions and showed that *in vitro* co-incubation of sialylated HMO and extraintestinal *enterotoxigenic E. coli* (ETEC), or *uropathogenic E. coli* (UPEC), inhibit fimbriae-mediated erythrocyte agglutination. Similarly, Lin et al. (2014) also demonstrated that sialylated HMO protect bladder cells against invasion and internalization by UPEC strain CFT073.

The antiadhesive and antimicrobial properties of HMO expand beyond bacterial pathogens and also apply to parasites, fungi and viruses. *Entamoeba histolytica* (*E. histolytica*) for example, expresses a surface lectin to facilitate its attachment and phagocytosis into intestinal epithelia. Exposure to pHMO significantly detached already-bound *E. histolytica* (by 80%) to epithelial cells but HMO were not successful in detaching *G. lambli.* Those results imply that the mechanism employed is specific to *E. histolytica* (Jantscher-Krenn et al., 2012). Binding of the fungus *candida albicans* to epithelial cells often contributes to the development of NEC and is disrupted by fucosylated HMO (Brassart et al., 1991). In addition, HMO display antimicrobial properties by reducing growth and development of the yeast cell in a dose-dependent manner (Gonia et al., 2015).

Various studies highlight the protective effects of HMO against viruses that employ glycans as a binding mechanism. The attachment of noroviruses, such as norwalk virus are blocked by milk from secretor mothers (Jiang et al., 2004; Marionneau et al., 2005). In addition, Morrow & Rangel (2004) demonstrated that mother's milk containing high concentrations of α1-2-fucosylated HMO reduced the occurrence of calicivirus diarrhea in the breast fed infants. In the more recent years, the effects of HMO on human immunodeficiency virus (HIV) have been elucidated. Several studies provide evidence that HMO reduce the occurrence of HIV transmission from mother to child by outcompeting the HIV glycoprotein (gp120) that binds to dendritic cells (Hong et al., 2009; Naarding et al., 2005). Breast milk of HIV-positive mothers has potent HIV inhibitory activity that is unique to human milk (Wahl et al., 2012, 2015). A cohort study demonstrated that the abundance of 3'SL was higher in the

milk of HIV-1 infected mothers compared to uninfected (Van Niekerk et al., 2014). The consumption of breast milk high in 2'FL was protective against mortality in HIV-exposed, uninfected (HEU) breast-fed children (Kuhn et al., 2015). Maternal HIV infection is thought to contribute to a disruption of the microbiome (Bender et al., 2016) and therefore reported effects of HMO in HEU infants may be in part due to the influence of HMO on the bacterial species of the infant microbiome. Once again, these findings confirm the multi-approach mechanism used by HMO to promote the infant's well-being.

1.1.4.3 Nutrients for the brain

Small or premature infants that are breast-fed have higher intelligent quotient (IQ) scores (Lucas et al., 1990, 1992) advanced learning abilities, a lower risk of behavioral problems such as attention-deficit hyperactivity-disorders (Park et al., 2014) and cognitive advantages compared to infants that are formula fed (Lucas et al., 1990). A more recent study demonstrated the long-term consequences of human capital and its correlation to breast-feeding. Those who breast-fed for a longer period of time showed an increase in IQ, years of schooling and income at 30 years of age (Victora et al., 2015). Development and cognition partially depend on poly-Sia containing glycoproteins and Sia-containing gangliosides (reviewed in Wang 2009). Sia concentrations in the brain more than double, a few months prior birth, until 2 years post birth (Svennerholm et al., 1989). Sialylated HMO contribute to the majority of Sia in human milk and is 2–3-fold higher than that from glycoproteins, or Sia from glycolipids (accounting for only ~1% of the total milk Sia) (Bing Wang et al., 2001). Post-mortem analysis on human neonates showed that breast-fed infants had significantly higher concentrations of protein bound, ganglioside bound, and total brain Sia, when compared to infants that were formula fed (Wang et al., 2003). Animal studies also showed that learning and memory increases when piglets are fed sow milk replacer supplemented with sialylated casein glycomacropeptide (Wang et al., 2007). Additional experiments in rodent models also contribute to the notion that dietary Sia is an essential nutrient for brain development (Carlson & House, 1986). Whether sialylated HMO directly provide the developing brain with Sia, remains to be investigated. Expanding research is shedding light on the function of the brain-gut-microbiota axis. Intestinal microbiome is thought to play a key role in early programming and regulation of the infant's neuro-immune system. Strong evidence indicates that through brain-gut communication the gut microbiome can critically influence brain function and stress responses (Cong et al., 2016, 2015). A recent analysis revealed mice fed sialylated bovine milk oligosaccharides (sBMO) had significantly elevated free Sia metabolites in their brains compared to non-treated mice. These findings were dependent on their microbiota composition and the presence of bacterium *B. fragilis,* known to degrade sialyllactose, and therefore increase free Sia (Charbonneau et al., 2016). Feeding

3'SL and 6'SL to mice helped support normal microbial communities and normal behavior responses during stress and anxiety-like behavioral tests and maintain normal numbers of DCX+ immature neurons (Tarr et al., 2015). Additional studies such as this one could provide further insight as to how HMO may contribute to increased brain Sia and superior brain cognition in breast-fed infants, through their effects on the gut microbiota-brain axis.

1.2 Human milk, immunity, and inflammation

1.2.1 Immunity overview

The immune system is divided into two categories, known as innate and adaptive immunity. In the likelihood of a threat to the host, the innate system provides the first line of defense and results in a response that is rapid, short-lived, and non-specific to a broad array of pathogens during acute inflammation (reviewed in Turvey & Broide 2010). The primary barriers of the innate system include mucosal tissues and cells such as granulocytes, dendritic cells (DC), natural killer (NK) cells, and macrophages. The actions of immune cells are reinforced by additional factors such as lysozymes, antimicrobial peptides, and cytokines. Within several days this initial phase of inflammation triggers the adaptive response. The threats to the host are further targeted with lymphocytes, such as B and T cells that recognize an array of foreign antigens to target the specific threats precisely in the region of the body where the threat is occurring (reviewed in Murphy Kenneth 2011; Levy & Wynn 2014). T cells are an integral component of the adaptive immune response due to their ability to recognize antigens presented in MHC class I and II. Antigen recognition leads to cytokine production that in turn stimulates phagocytic cells to target the presented threats. B cells also interact with antigens. They proliferate and differentiate into plasma cells, to produce antibodies, to then eliminate the foreign antigens. DC populate different tissues and are antigen presenting cells (APC) that capture and display antigens to lymphocytes (Levy & Wynn, 2014; Kenneth, 2011; Turvey & Broide, 2010). The exposure to this response results in an enhanced function of the innate immune system, which then forms a “memory” to facilitate a faster adaptive response upon re-infection, referred to as “trained immunity” (Netea et al., 2011).

1.2.2 Impact of breast-feeding on immune development

Human milk boasts a variety of bioactive components such as lipids, proteins, and carbohydrates that shield the infant from pathogenic infections (Andreas et al., 2015; and described in 1.1.4.2), regulate microbial composition (Walker & Iyengar 2015; and described in 1.1.4.1), and promote immune function (Verhasselt, 2010). The development of the infant’s immature immune system is further enriched by several classic innate immune effectors,

such as antibodies (Levy, 2007; Thomas et al., 2004), cytokines (Garofalo, 2010; Kverka et al., 2007), and cells (Ichikawa et al., 2003; Speer et al., 1985), which are all present in human milk. The newborn infant possesses a functionally naïve immune system which varies from that of the adult in terms of the quality and quantity of immune components (Belderbos et al., 2012; Gibbons et al., 2014; Netea et al., 2011; Siegrist et al., 1998). These known differences between adults and infants result in different innate and adaptive immune responses (Levy & Wynn, 2014). Trained immunity (described 1.2.1) is evident in human neonates, less than a month age and may be critical for early life survival of the host, possibly influencing the outcome of infections, allergies, and chronic inflammatory diseases later in life (Kindt et al., 2007).

Exclusive breast-feeding for more than 6 months influences the maturity of the immune system (Volman et al., 2008; West et al., 2010) and lessens the prevalence and severity of infectious diseases, compared to formula-feeding (Golding et al., 1997; Stuebe & Schwarz, 2010). In addition, there is evidence that breast-feeding decreases the prevalence of allergies, asthma, inflammatory bowel disease, celiac disease, type 1 diabetes and both acute lymphoblastic and myeloblastic leukemias (Donovan & Comstock, 2016; Li et al., 2014; Munblit & Verhasselt, 2016). The lower morbidity and mortality rates associated with breast-feeding maybe attributed to human milk constituents that contribute to the innate immune defense and factors that promote immune development (Kelly & Coutts, 2000; Turfkruyer & Verhasselt, 2015; Verhasselt, 2010). Previous studies have shown that formula-fed (FF) infants have a fewer percentage of NK cells and a higher CD4:CD8 ratio in peripheral blood mononuclear cells (PBMC) (Andersson et al., 2009; Hawkes et al., 1999), more Th cells (Carver et al., 1991), and a stronger lymphocyte proliferation in response to mitogen (Juto et al., 1982; Stephens et al., 1986), when compared to breast-fed (BF) infants. The composition of immune cells observed amongst BF infants are thought to contribute to a healthy immune system and may be attributed in part to specific components in human breast milk, that are devoid in formula (Pickering et al., 1998).

1.2.2.1 Immunomodulatory properties of human milk oligosaccharides

HMO represent a key bioactive component of milk and play a role in neonatal immune system, while providing protection to the host and mediating immune responses by engaging a variety of mechanisms (Kulinich & Liu, 2016; Newburg & He, 2015). Limited data has been generated to reveal the immunological implications of the direct interactions of HMO with immune cells of the GI tract (Mcguire et al., 2017). *In vitro* studies using HMO and intestinal cells lines have been used to explore the effect of HMO on immune-related gene expression. In the human colon adenocarcinoma cell line (H-T29 cells) HMO increase the expression of various chemokines (Lane et al., 2013) and reduce genes associated with intestinal

inflammation in T84 colonic adenocarcinoma cell line and HCT8 colon carcinoma cell line (He et al., 2014). Another study in human normal fetal intestinal epithelial cells (H4 cells) resulted in attenuated expression of *IL-8*, MIP-3α and MCP-1, induced by *TNF-α* and *IL-1β*, when exposed to HMO. In addition, similar results were observed for the expression of pathogen-induced *IL-8* and MCP-1, when H4 cells were exposed to HMO (Newburg et al., 2016). *In vivo* studies showed that when pigs were fed mixtures of HMO (consisting of mostly 2'FL and LNnT) their ileal tissues resulted in greater *IFN-γ* secretions by THP-1 human monocytic cell line and higher *IL-1*0 mRNA, than that from pigs fed formula (Li et al., 2014). An additional feeding study showed that mice fed dextran conjugated HMO Lacto-N-fucopentaose III (LNFP III) and lacto-N-neo-tetraose (LNnT) resulted in an expansion of a subclass of peritoneal macrophages that suppressed naïve CD4+ T-cell responses. This led to a reduction in pro-inflammatory cytokines and thereby generated an anti-inflammatory environment (Atochina et al., 2001; Terrazas et al., 2001). Atochina & Harn (2005) followed up with an *in vitro* study and determined that LNFP III stimulated macrophages to activate NK cells, leading to an increase in prostaglandin E_2, interleukin-10 (*IL-10*) and *TNF-α* secretions (Atochina and Harn, 2005).

When undigested HMO are found intact within the bloodstream of the infant (described in 1.1.3.5), they are thought to perhaps directly affect systemic immune cells. For example, Eiwegger et al. (2004) demonstrated the capability of acidic HMO to stimulate interleukin-13 (*IL-1*3) in CD8+ cells and interferon-gamma (*INF-γ*) in CD4+ and CD8+ T-cell originating from cord blood (Eiwegger et al., 2004). Similarly, acidic HMO were able to induce the anti-inflammatory cytokine, *IL-1*0 in cord blood mononuclear cells. A study by Comstock et al. (2014), using PBMC isolated from 10-day old pigs, identified sialylated HMO as stimulators of *IL-1*0 and cell proliferation was increased after exposure to various mixtures of HMO. These findings indicate that HMO can possibly promote lymphocyte maturation and a balanced cytokine production in newborn infants.

Human milk may also have the capacity to regulate immune responses to precisely combat allergy epidemics (Munblit & Verhasselt 2016). Recent data suggest that milk with higher concentrations of LNFP III are associated with the lack of development of bovine milk allergy (Seppo et al., 2016). Another study by Sprenger et al. (2016) showed that infants with a high hereditary risk for allergies, born by C-section and fed breast milk with FUT2-dependent milk oligosaccharides, may be at lower risk of developing IgE-associated eczema at 2 years. In addition, *in vitro* results indicate that sialylated HMO also suppressed the Th-2-type cytokine production of *IL-4*, in a subset of lymphocytes from adult patients with peanut allergies (Eiwegger et al., 2010). These results highlight the ability of HMO to modulate allergen-specific immune responses.

1.2.2.2 Immune cell protein-glycan interactions

HMO have been shown to bind to distinctive carbohydrate binding proteins on immune cells that recognize specific glycan motifs leading to diverse immune-related actions (see Table 1.1). These carbohydrate binding motifs on cells are known as lectins (Gabius et al., 2011; Hart & Akimoto, 2009; Schnaar, 2015; Taylor & Drickamer, 2014) and are categorized according to their carbohydrate recognition domains (CRD). For example, recent studies suggest that HMO may play a role in infant immunity (Noll et al., 2016) by interacting with S-type lectins such as galectins, a family of proteins that specifically bind to Gal-rich glycans. HMO that can contain β1-3- or β1-4-linked Gal at their non-reducing ends could potentially interact with galectins, which are implicated in immune regulation, by mediating activated T-cell apoptosis (Barondes et al., 1994; Cummings & Liu, 2009).

The C-type lectin DC-specific intercellular adhesion molecule 3 grabbing non-integrin (DC-SIGN) has a CRD specific to fucose (Becker & Lowe, 2003; Bogoevska et al., 2006), and interactions with fucosylated ligands, contribute to immune tolerance (Conde et al., 2015; Geijtenbeek et al., 2004; Steinman et al., 2003). The transfer of HIV-1 to CD4+ T lymphocytes is inhibited by the binding of Lewis antigen glycans to DC-SIGN and since HMO carry one or more Lewis antigen epitopes, they are thought to perhaps play a role in preventing HIV transfer from mother to infant (Bode et al., 2012).

Selectins are also C-type lectins and play a role in regulating immune cells. The selectins expressed on activated endothelial cells recognize carbohydrate structures of leukocytes and during an inflammatory response, leukocytes adhere to these selectins to travel from the blood stream through the vascular endothelium into lymphatic tissue or sites of inflammation (Springer, 1994). The ligands of selectins such as fucose and sialic acids are all components of HMO (Bevilacqua & Nelson, 1993; Huang et al., 2002; Ley, 2003a; Lühn & Wild, 2012). Due to the structural similarities, HMO can bind selectins to alter the immune system by affecting cell populations and functions mediated by selectin interactions (Bode & Kunz, et al., 2004; Bode & Rudloff, et al., 2004; Comstock et al., 2014; Rudloff et al., 2002; Schumacher et al., 2006). For example, Glycosylation-dependent Cell Adhesion Molecule (GlyCAM-1) is a ligand for L-selectin and is expressed by naïve and central memory T-cells. These cells enter the lymph nodes via their L-selectins binding to GlyCAM-1 on high endothelial venules (Sallusto et al., 1999). Sialylated HMO (e.g. 3'SL and 3'sialyl-3-fucosyl-lactose) prevent excessive infiltration of leukocytes and therefore severe tissue damage, by reducing leukocyte adhesion and rolling in tumor necrosis factor alpha (*TNF-α*) activated human endothelial cells (Bode & Kunz et al., 2004).

In circulating blood, selectins are necessary to form large reactive subpopulations of neutrophils, known as platelet-neutrophil complex (PNC). Glycoconjugate ligands present on neutrophils are recognized by selectins on activated platelets and binding results in an increased expression of adhesion molecules (including β2 integrin CD11b/CD18) leading to a greater incidence of phagocytosis and production of reactive oxygen species (ROS) by neutrophils (Larsen et al., 1989; Peters et al., 1999). Sialylated HMO have been shown to reduce PNC formation and the correlated β2 integrin expression (Bode & Rudloff et al., 2004). Persistent selectin inhibition could compromise the innate immune system (Ley, 2003b) but sialylated HMO targeting both leukocyte trafficking and PNC formation, along with their pro-inflammatory properties, could reduce the incidence of inflammatory diseases in breast-fed infants (Bode & Rudloff et al., 2004; Schumacher et al., 2006).

Sialic acid binding Ig-like lectins (Siglecs) are frequently observed on the surface of immune cells and are I-type lectins that specifically bind to sialic acid containing ligands (Bochner & Zimmermann, 2015; Crocker & Varki, 2001; Macauley et al., 2014). Siglecs are thought to regulate the functions of cells in the innate and adaptive immune systems through recognition of their sialic acid ligands, thus discriminating between 'self' and 'non-self' (Macauley & Paulson, 2013). Siglecs are expressed on B cells, NK cells, neutrophils, basophils, eosinophils, dendritic cells, monocytes, and macrophages. They can be categorized into two groups: those that are conserved across mammals and CD33-related Siglecs (variable across mammals). The cytoplasmic domain of most Siglecs have (ITIMs) and signal negatively via recruitment of tyrosine phosphatases such as SHP-1 and SHP-2. Some act as activating receptors via a positively charged amino acid in their transmembrane region through the recruitment of SYK kinase, after associating with the tyrosine-based activation motif (ITAM) (Hart & Akimoto, 2009; Varki & Gagneux, 2012). Koliwer-Brandl et al. (2011) have shown that DSLNT inhibited siglec-4 but not siglec-2 binding to glycosylated surfaces. The capability of HMO to beneficially shape the gut microbiome (described in 1.1.4.1), in combination with their observed interactions with pathogens (described in 1.1.4.2) and systemic immune cells, provides evidence for HMO as major drivers and modulators of immune function for the breast-fed infant.

Table 1.1 Possible interactions of HMO with known protein-glycan structures on immune cells. Adapted from (Bode, 2006).

Lectin	Possible HMO Interactions
Selectins	Fucosylated and Sialylated structures
DC-SIGN	Fucosylated structures
Galectins	β-galactosides (HMO backbone)
Siglecs	Sialylated structures
Others?	

1.2.3 Macrophages and inflammation

Before immune cells are released directly into systemic circulation, they mature in the bone marrow, with the exception of T lymphocytes that undergo maturation in the thymus. During fetal development, haematopoiesis originates in the yolk sac, passes through the fetal liver and at birth ends up in the bone marrow (Udalova et al., 2016), where both red and white blood cells (WBC), also known as leukocytes, are produced from hematopoietic stem cells (HSC). Multipotent HSC differentiate into common myeloid or lymphoid progenitor cells and once they have lost the ability to self-renew, they give rise to specific cell types. T cells, NK cells and B cells, differentiate from lymphoid progenitor cells. Erythrocytes, megakaryocyte progenitor cells (which gives rise to platelets), as well as dendritic cells, monocytes and all granulocytes: basophils, eosinophils and neutrophils, all originate from myeloid progenitor cells. Circulating monocytes leave the blood stream after a few hours and migrate into tissues, where they differentiate into tissue-specific macrophages (Udalova et al., 2016) .

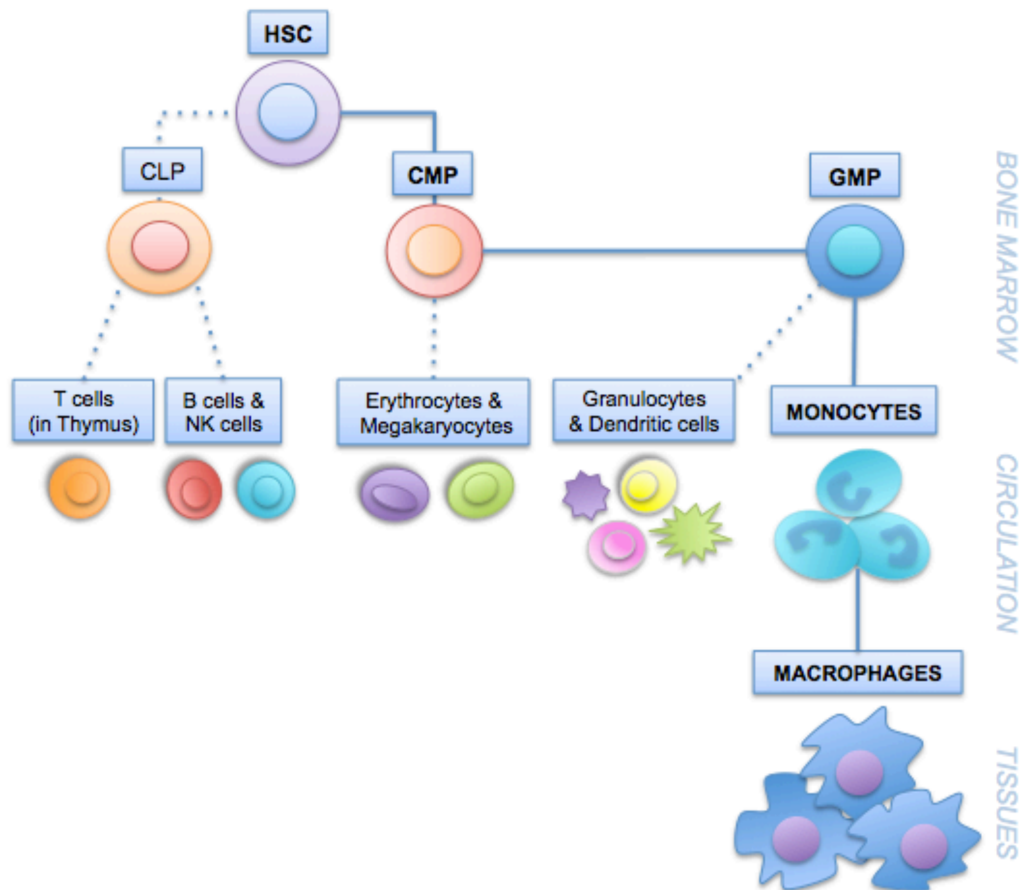


Figure 1.7 Haematopoiesis in the adult bone marrow.
The bone marrow is the predominant site of haematopoiesis, which relies on self-renewing haematopoietic stem cells (HSC) constantly generating progenitors. The lymphoid lineage derives from common lymphoid progenitor (CLP), which gives rise to T-cells, B-cells and NK cells. The myeloid lineage derives from the common myeloid progenitor (CMP), which gives rise to erythrocytes and megakaryocytes, as well as the granulocyte–macrophage progenitor (GMP). Granulocytes and dendritic cells arise from additional precursors not shown. Monocytes circulate in the blood and upon tissue entry can differentiate into macrophages. Figure adapted from (Udalova et al. 2016)**.**

In order to fulfill their role in immune surveillance, macrophages have ability to sense a broad range of stimuli and are considered the frontline soldiers of innate immunity, with the ability to fight off threats, mediate inflammation and sustain tissue integrity (Martinez & Gordon, 2014; Novak & Koh, 2013; Sica & Mantovani, 2012). Their activation involves a complex mechanism towards two opposite conditions, known as classical (M1) and alternative (M2) activation (Martinez & Gordon, 2014; Sica & Mantovani, 2012). The M1 activation is induced by bacterial cell wall components such as lipopolysaccharides (LPS) or lipid A, lipoproteins, and cytokines, such as tumor necrosis factor alpha (*TNF-α*) and interferon gamma (*IFN-γ*). The M1 macrophages are characterized as "pro-inflammatory" and produce nitric oxide (NO) in addition to secreting inflammatory cytokines (Benoit et al., 2008; Martinez & Gordon, 2014;

Murray et al., 2014; Sica & Mantovani, 2012). Although M2 macrophages are thought to contrast M1 activated macrophages and widely termed as anti-inflammatory (Morris et al., 2011; Pollard, 2009), some findings question whether the macrophage activation of M1 and M2 macrophages *in vivo* is clearly defined (Roszer, 2015). The M2 macrophages are activated by various factors such as parasites, fungi, interleukin-4 (*IL-4*), *IL-13*, *IL-10*, tumor growth factor beta (*TGF-β*). In response M2 macrophages are capable of phagocytosis and produce *IL-10,* extracellular matrix components (ECM) and chemotactic factors (Bohlson et al., 2014; Fuentes et al., 2010; Murray et al., 2014). Maintaining the M2-like state of resident macrophages in joints, liver, or adipose tissues for example, would help diminish the production of inflammatory mediators in those sites and be an advantageous method to treat metabolic diseases (Glass & Olefsky, 2012; Osborn & Olefsky, 2012; Roszer, 2015).

1.2.3.1 Recognition of LPS by TLR4 on macrophages

Macrophages have the ability to recognize and respond to pathogens due to the expressed the pattern recognition receptor (PRR), which are key elements in the innate immune system and known as Toll-like receptors (TLR). Humans have 10 different TLR whereas mice have 12 and each one has a particular molecular activation trigger (Kawai & Akira, 2011; Trinchieri & Sher, 2007). More specifically, the TLR-4 has a high affinity for lipopolysaccharides (LPS), one of the most common immuno-stimulants used in research to activate and study M1-like macrophages. Also known as endotoxins, they are located in the outer cell membrane of most gram-negative bacteria and consist of a hydrophilic heteropolysaccharide covalently linked to a lipid component, known as lipid A (Rietschel et al., 1994). Upon interaction at the cell surface of macrophages, LPS bound to LPS binding protein (LBP) is transferred to CD14 and the lipid A component of LPS is recognized by TLR4 and its co-receptor MD-2 (Figure 1.8) (Kawai & Akira, 2010). LPS recognition by TLR4 triggers two distinct intracellular signaling cascades, the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway and the TIR-domain-containing adaptor protein inducing interferon-β (TRIF)-dependent pathway (Yong Chen Lu et al., 2008), resulting in the transcriptional up- or down-regulation of genes leading to the downstream secretion of cytokines, chemokines, and antimicrobial effector mechanisms (Figure 1.8) (Nau et al., 2002).

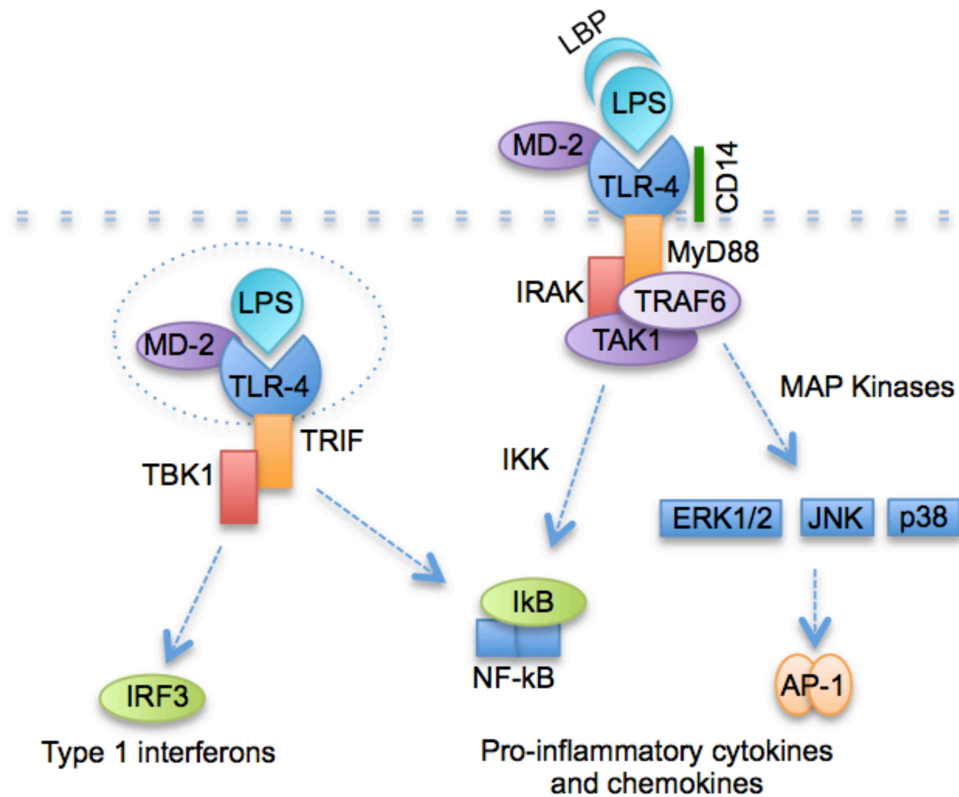


Figure 1.8 Binding of LPS to TLR4 in macrophages.
Activation of the MyD88-dependent and TRIF-dependent pathways leads to the recruitment of adaptor proteins and promotes the activation of NF-κB, AP-1 and IRF3 transcription factors, for further transcription of genes encoding for pro-inflammatory mediators. Modified from (Lu et al., 2008; Maeshima & Fernandez, 2013).

The adaptor protein MyD88 is recruited and associates with *IL-1R*-associated kinases (IRAK), TNF receptor-associated factor 6 (TRAF6), and the transforming growth factor-β-activated kinase 1 (TAK1) complex, to in turn activate MAPK and IKK pathways (Kawai & Akira, 2010). IMAPK are intracellular serine/threonine kinases and are comprised of p38, MAPK, JNK, and extracellular signal-regulated kinase1/2 (ERK1/2). They activate AP-1 and NF-κB transcription factors, which regulate the transcription of pro-inflammatory cytokines and chemokines (Chan et al., 2001). In the unstimulated state, the heterodimeric NF-κB molecule is bound to inhibitory proteins, IκB. Upon LPS-stimulation, IKK phosphorylates serine residues of IκB, which are ubiquitinated and targeted by proteasomal degradation (Karin & Delhase, 2000), to release NF-κB and translocates to the nucleus to bind promoter regions of genes that encode for pro-inflammatory cytokines (Ulevitch & Tobias, 1995). NF-κB can also be activated by the TRIF-dependent pathway (Barton & Kagan, 2009). This occurs once TLR4 and MD-2 complex are internalized into endosomes. TRIF initiates the second pathway to activate the transcription factor interferon regulatory factor 3 (IRF3), leading to the *IFN-β* expression (Kawai et al., 2001) and activation of the JAK-STAT1 intracellular signaling pathway. This induces STAT1-dependent genes (Ohmori & Hamilton,

2001; Toshchakov et al., 2002a) to further promote expression of pro-inflammatory cytokines.

1.2.3.2 Effects of HMO on LPS-stimulated macrophages

Several studies have indicated that HMO possess anti-inflammatory effects on different cells of the immune system (described in 1.2). Preliminary results, generated in the lab of Dr. Lars Bode and described in the master's thesis titled "Human Milk Oligosaccharides Modulate Macrophage Response" (Szyszka, 2014), reports the anti-inflammatory effects of HMO on LPS-stimulated macrophages, *in vitro*. The anti-inflammatory effects of HMO were determined by assessing the inhibition of pro-inflammatory cytokines. Results revealed that the addition of pooled (pHMO; dark blue bar), neutral (nHMO; light blue bar), and acidic HMO (aHMO; light blue bar) reduced mRNA expression of *IL-6* and *IL-1β* (Figure 1.9) in the LPS-stimulated mouse macrophage cell line, RAW 264.7.

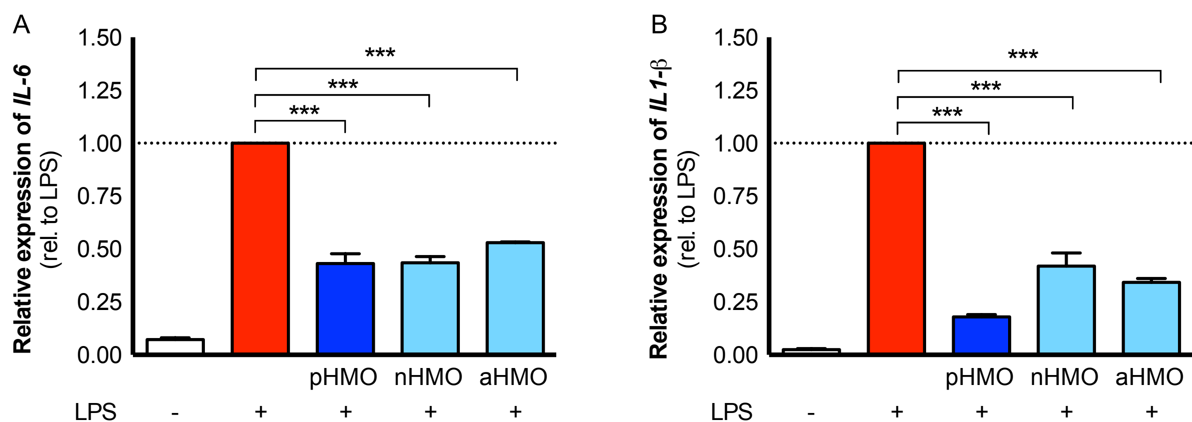


Figure 1.9 Pooled HMO (pHMO), neutral (nHMO), and acidic (aHMO) reduce relative expression of *IL-6* (A) and *IL-1β* (B) in LPS-activated RAW 264.7 macrophages.
Total RNA was isolated and qRT-PCR analysis was conducted to measure mRNA levels of *IL-6* and *IL-1*β. Data shown are the fold induction of gene expression relative to 10 ng/mL LPS. One-way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (*** $p<0.001$). Bars represent the mean ± SEM; n=2. (Graph modified from Szyszka, 2014)

There are still many questions that remain to be answered in regard to the anti-inflammatory properties of HMO. Whether the observed effects on macrophages are structure specific and attributed to a single HMO structure has yet to be determined. In addition, it is not known if HMO have an effect on other markers of inflammation and if the described results are cell-line dependent. Furthermore, the question of how HMO induce their cytokine expression in macrophages remains unsolved. The translational aspect of these findings (*in vivo*) for the therapeutic application of HMO, in the context of chronic diseases, still needs to be explored.

1.2.4 Role of macrophages in rheumatoid arthritis

Macrophages are highly versatile cells that can respond rapidly to many stimuli (previously described in 1.2.3), making them potentially very important in the pathogenesis of many diseases. Although rheumatoid arthritis (RA) is not solely macrophage-dependent, macrophages play a key role in the development of RA (Figure 1.10). They are of critical importance because they generate cytokines and recruit immune cells that heighten inflammation and contribute to the damaging effects on cartilage and bone (reviewed by Udalova et al., 2016). Biopsies of synovial membranes of patients with RA revealed that diseased tissue contains greater numbers of macrophages when compared to tissue from healthy patients with normal synovial membranes. The observed synovial lining and sub-lining tissue (Janossy et al., 1981) also contain a dramatically increased number of macrophages that are thought to drive T-cell infiltration via antigen presentation. This action in the synovial membranes of patients with RA triggers B-cell infiltration, immunoglobulin production, synovial fibroblast activation, and inflammatory secretions. In turn, this creates a positive feedback effect on macrophages, leading to an environment of chronic or persistent inflammation (Figure 1.10). Osteoclasts, which are multinucleated monocyte-macrophage derivatives, reside within bone tissue and cause severe bone degradation and erosion when recruited and activated by the described inflammatory process (Blair, 1998).

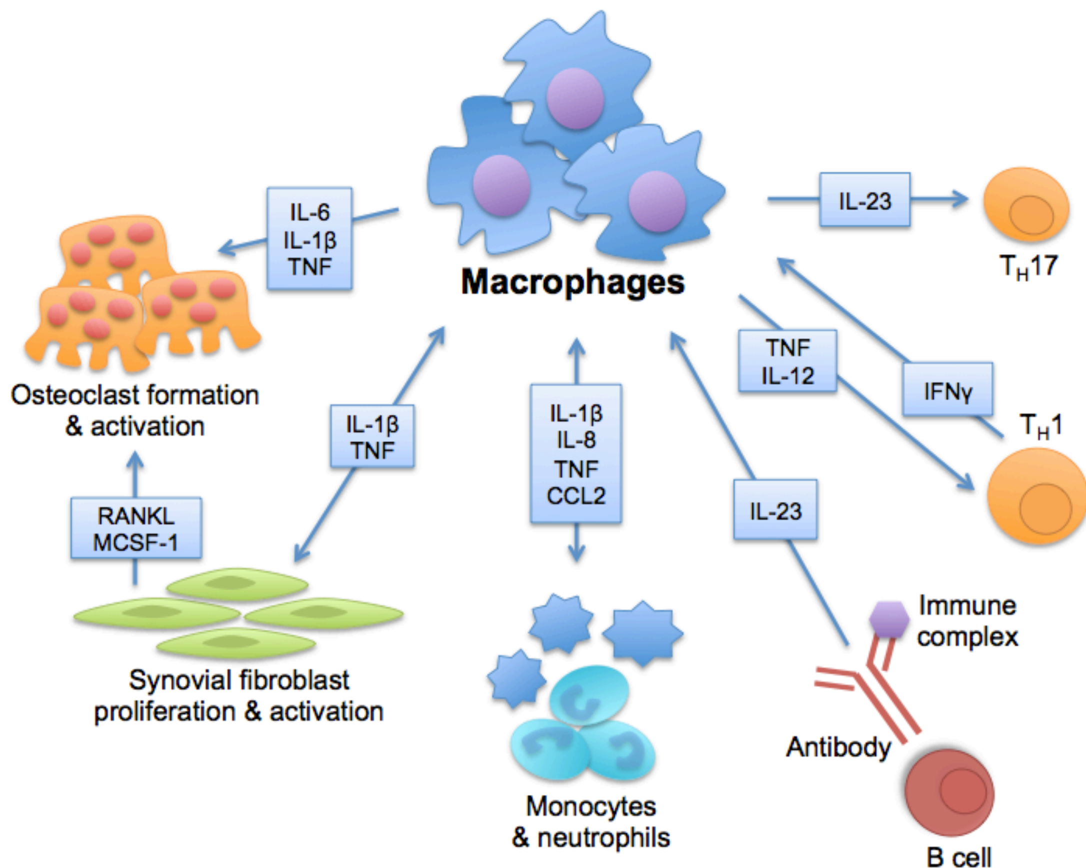


Figure 1.10 The role of macrophages in RA

Macrophages promote inflammation by producing cytokines to recruit additional immune cells and activate fibroblasts. In turn, fibroblasts and macrophages induce osteoclast differentiation, which contributes to bone degradation. In addition, macrophages are activated by cell–cell contact, cytokines from T-cells, fibroblasts and other innate immune cells, as well as immune complexes and antigens. Figure adapted from (Udalova et al., 2016).

Acute inflammation is also characterized by the onset of a pro-inflammatory phase, but differs in such that it is followed by a resolution phase. This second phase of acute inflammation is set in motion by the arrangement of specific factors that provide pro-resolving actions to promote repair and homeostasis (reviewed in Perretti et al., 2017). The complex resolution mechanisms in inflammatory rheumatoid arthritis are still extensively under investigation and studies in mice have described the involvement of several anti-pro-resolving pathways. Genetically modified mice lacking either a high-affinity receptor for LXA, RvD1, and AnxA1, known as *N*-formyl peptide receptor 2 (ALX/FPR2), exhibit aggravated tissue damage and arthritic disease severity (Dufton et al., 2010; Kronke et al., 2009). In a collagen-induced arthritis model, 5-LOX-null mice, resulted in a blocked (cyclooxygenase-2) COX2 pathway activity and exhibited an intensified and prolonged infectious arthritis. Their findings demonstrated that arthritis can be resolved by the prostaglandin E_2 (PGE_2) mediated production of LXA (Chan & Moore, 2010). Current therapeutic agents for persistent

inflammation in a given tissue are anti-inflammatory in their actions, but yet are often still inadequate to fully undertake the pathology of rheumatic diseases.

The estimated prevalence of RA worldwide is believed to range from 1% to 3% (Dieppe, 2002; Sacks et al., 2010). RA has been shown to lower the quality of life in both the industrialized and developing worlds and eventually results in premature death and disability (Brooks, 2006). Although the onset of RA is highest among those over sixty years of age, the etiology is unknown but is believed to result from a combination of genetic and environmental factors (Dieppe, 2002). The incidence of RA is usually two to three times higher in women than in men, however the majority of patients with RA go into remission during pregnancy (de Man et al., 2008). Although many experience flares during the postpartum period (Barrett et al., 1999), population based studies have found that RA is less common among women who breast-feed (Colebatch & Edwards, 2011; Doran et al., 2004; Jaakkola & Gissler, 2005; Pope et al., 1999).

Juvenile rheumatoid arthritis (JRA) is the most common type of arthritis found in children and approximately 1 in every 1000 children world-wide is thought to have JRA. Due to under-diagnosis and the lack of a specific diagnostic test, the reported incidence and prevalence of JRA varies widely and the available data are likely to underestimate the true incidence and prevalence of JRA (Huang, 2012; Shen et al., 2013). Breast-feeding is thought to be associated with a reduced risk of developing JRA. A case-control study by Mason et al. (1995) reported that those who have had JRA are less likely to have been breast-fed than controls. Another case study by Young et al. (2007) observed that children who were HLA-DR4 negative and breast-fed for over 3 months did not develop JRA. Taken together, the available evidence suggest that breast-feeding may have a protective effect on the development of JRA. Further research is necessary to investigate correlations and the mechanistic effects of breast-feeding on JRA.

Currently, the greatest challenge in treating patients with RA is predicting which patients will respond to which specific medication. In addition to the grueling side-effects of treatments, the most used medications Methotrexate and Leflunomide must be avoided during lactation (Sammaritano & Bermas, 2014). Although research is ongoing, there has been no practical application of the advances made to improve the treatment of RA.

1.3 Objective

Data generated by Szyszka (2014) reports that HMO have inhibitory effects on the secretion of pro-inflammatory cytokines in LPS-activated RAW 264.7 macrophages (also described in 1.2.3.2). These results provide first evidence for the potential use of HMO as anti-

inflammatory compounds and merits additional investigation on the role of HMO on macrophages. Based on these preliminary results, specific objectives were established to further explore the effects of HMO in the context of chronic low-grade inflammation. The objectives of this project were:

1. To identify the specific HMO structures responsible for anti-inflammatory effects in macrophages.

Prior results provided no indication as to which of the 150 different HMO is responsible for reducing inflammatory markers in macrophages. Therefore, fractions containing defined HMO were tested and results were further confirmed in macrophages, by testing individual chemically synthesized HMO.

2. To define the underlying molecular mechanisms employed by HMO using various macrophage models

The anti-inflammatory effects of HMO on cytokine gene expression level in macrophages were verified *ex vivo* with primary macrophages isolated from mice and also confirmed in human macrophages. In addition, the effects of HMO on LPS-induced expression of inflammatory mediators other than *IL-6* and *IL-1β* were investigated. This led to assessing possible glycan-glycan interactions between LPS and HMO at the TLR4 receptor, as a mechanism to prevent cytokine expression. To provide further insight into HMO-affected signaling pathways, exogenous stimulants other than LPS were tested to induce a low grade chronic inflammation. Finally, the identification of affected macrophage signaling pathways and the expression of receptors involved were determined.

3. To determine whether or not the observed anti-inflammatory effects of HMO translate to *in vivo* efficacy in a mouse model of rheumatoid arthritis (RA)

Since macrophages have a crucial role in the initiation of inflammatory responses, the reported effects of HMO may have the potential to prevent chronic low-grade inflammatory diseases. Macrophages play a key role in the pathogenesis of RA and therefore testing HMO in a mouse model for RA can provide information on the physiological effects of HMO *in vivo,* for their application as anti-inflammatory compounds.

2 Materials and Methods

2.1 Materials

Table 2.1 Mice used for experiments
Mice were kept in a pathogen-free animal facility. Mice were provided with food and water *ad libitum* at a 12 h light/dark cycle. All procedures were performed according to the Institutional Animal Care and Use Committee (IACUC) of the University of California San Diego.

Mouse Line	Provider	Reference
C57BL/6J	Own breeding and keeping	
ST3Gal-IV KO	Own breeding and keeping	(Ellies et al., 2002)
Sialoadhesin KO	Provided by Dr. Victor Nizet	(Oetke et al., 2006)
Siglec E KO	Provided by Dr. Ajit Varki	(Brinkman-Van der Linden
BAI-1 KO	Provided by Dr. Peter Ernst	(Koh et al., 2001)
Balb/c	Own breeding and keeping	

Table 2.2 Cells used for experiments

Cells	Reference
Raw 264.7	ATCC TIB-71
THP-1	ATCC TIB-202
HEK293T	ATCC CRL-3216
Bone marrow derived macrophages (BMDM)	Primary cell isolation within the lab

Table 2.3 Chemicals used for experiments

Chemical and reagents	Company	Catalog number
2-Mercaptoethanol	Sigma-Aldrich	M3148
Accell Delivery Media	GE Dharmacon	B-005000
Acetic acid, glacial	Thermo Fisher Scientific	A507-212
Anthranilamide	Sigma-Aldrich	A-9397
Bio-Gel®P-2 Gel	BIO-RAD	150-4114
Bovine Serum Albumin	Sigma-Aldrich	A3059
Cell Dissociation Buffer	Life Technologies	13151-014
Chloroform	Thermo Fisher Scientific	C606-1
Dimethyl sulfoxide	Sigma-Aldrich	D2650
DMEM	Life Technologies (Gibco)	11965-092
DMEM/F12	Sigma-Aldrich	51445C
Ethanol 190 proof	VWR International	V1105M
Ethanol 200 proof	Sigma-Aldrich	E7023-500ML
Fetal Bovine Serum	Gemini Bio-Products	100-106
Methanol	Thermo Fisher Scientific	A41220
MCSF	Shenandoah	200-08
PBS + $CaCl_2$ + $MgCl_2$	Life Technologies (Gibco)	14040-133
PBS	Life Technologies (Gibco)	14190-144

PageRulerPrestained Protein Ladder	Thermo Fisher Scientific	26619
Penicillin-Streptomycin	Life Technologies (Gibco)	15140-122
QAE Sephadex A-25 chloride	Sigma-Aldrich	Q25120
RPMI 1640 + Hepes	Life Technologies (Gibco)	22400121
siRNA Buffer	GE Dharmacon	B-002000-UB-100
Sodium Chloride	Thermo Fisher Scientific	BP358-212
Sodium Cyanoborohydride Reagent	Sigma-Aldrich	156159-10G
Sodium deoxycholate	Sigma-Aldrich	D6750-100G
Sodium dodecyl sulfate	Sigma-Aldrich	L3771
Sulfuric Acid	Thermo Fisher Scientific	A300-212
Stripping Buffer (PVDF 5x)	LI-COR	928-40032
Tris	BIO-RAD	161-0719
TRIzol® Reagent	Life Technologies (Ambion)	15596026
Trypsin EDTA	Corning Cellgro	25-052-CI
UltraPure™ Distilled Water	Life Technologies (Invitrogen)	10977-015
Water HPLC Grade Submicron Filtered	Thermo Fisher Scientific	W5-4

BIO-RAD (Hercules, CA, USA), Corning Cellgro (Tewksbury, MA, USA), Life Technologies (Carlsbad, CA, USA), Gemini Bio-Products (West Sacramento, CA, USA), LI-COR (Lincoln, NE, USA), Roche (Basel, Schweiz), EMD Millipore (Darmstadt, Deutschland), Sigma-Aldrich (St. Louis, MO, USA), Thermo Fisher Scientific (Waltham, MA, USA), VWR International (Radnor, PA, USA)

Table 2.4. Consumables used for experiments

Consumables	Company	Catalog number
1.5 mL Safe-Lock microcentrifuge tubes,	Eppendorf	22363204
3mL Syringe, Luer-Lock Tip	Covidien	1180300777
6-Well Cell Culture Plates	Genesee Scientific	25-105
10 cm² petri dishes	Thermo-Fisher Scientific	174888
10 cm² plates	Thermo-Fisher Scientific	150318
10 mL Syringe Luer-Lok Tip	BD Biosciences	309604
12-Well Cell Culture Plates	Genesee Scientific	25-106
24-Well Cell Culture Plates	Genesee Scientific	25-107
50mL Reagent Reservoirs	Corning Incorporated	4870
60mL Syringe, Luer-Lok™ Tip	BD Biosciences	309653
96 well plates (polystyrene, high bind)	Sigma-Aldrich	CLS9018
500mL Vacuum Filter System, 0.22µm	Corning Incorporated	431097
Cell counting chamber slides	Life Technologies	C10283
colorpHast® Indicator Strips	EMD Millipore	9578
Cryo 1°C Freezing Container	Thermo Fisher Scientific	5100-0001
Cryogenic Vial 1.2 mL	Corning Incorporated	430487
Deep Well plates 2mL	Axygen Scientific	P-2ML-SQ-C
Detoxi-GelTM Endotoxin Removing	Thermo-Fisher Scientific	20344
HyperSEB C18 Columns	Thermo Scientific	60300-422
HyperSEB Hyper Carb Columns	Thermo Scientific	60302-602
HyperSEB Silica Columns	Thermo Scientific	60300-482
KAPA SYBR Fast qPCR Kit Master Mix (2x)	KAPA Biosystems	KK4602
iScript cDNA Synthesis Kit	Bio Rad	170-8891
Optical Flat 8-Cap Strips for PCR Tubes	Bio Rad	TCS-0803

Microseal "B" adhesive seals	Bio Rad	MSB-1001
Mortar and Pestle	VWR	470148-960 (KT)
Multiplate® PCR Plates Low 96-well Clear	Bio Rad	MLL9601
Pierce® BCA Protein Assay Kit	Thermo-Fisher Scientific	23225
Precision Glide Needle 25G	BD	305122
RNase AWAY	Molecular BioProducts	7000
RNeasy® Mini Kit (50)	QIAGEN	74104
Round Bottom 96 Well Plate	Thermo Fisher Scientific	12 565 505
Single Edge Industrial Razor Blade	VWR	55411-055
SnapStrip® 8-Strip 0.2mL PCR Tubes	BioExpress	T-3035-1
Syringe Filter Unit, 0.22 µm	Merck Millipore	SLGV033RS
T-25 flasks	Thermo-Fisher Scientific	156367
T-75 flasks	Thermo-Fisher Scientific	156340
TipOne filter tip, 0.1-10/20 µL XL	USA Scientific	11203810
TipOne filter tip, 20 µL	USA Scientific	11201810
TipOne filter tip, 200 µL	USA Scientific	11208810
TipOne filter tip, 1000 µL	USA Scientific	11267810
XCell SureLock™, Mini-Cell	Life Technologies	E10001
XK 16/100 Column	GE Healthcare	28988947

Applied Biosystems (Foster City, CA, USA), BD Biosciences (San Jose, CA, USA), BioExpress (Kaysville, UT, USA), BIO-RAD (Hercules, CA, USA), Corning Incorporate (Tewksbury, MA, USA), Covidien (Mansfield, MA, USA), GE Healthcare Life Sciences (Pittsburg, PA, USA) Genesee Scientific (San Diego, CA, USA),Merck Millipore (Darmstadt, Deutschland), Life Technologies (Carlsbad, CA, USA), Molecular BioProducts, (San Diego, CA, USA), NEST (Rahway, NJ, USA), Sigma-Aldrich (St. Louis, MO, USA), Thermo Fisher Scientific (Waltham, MA, USA), USA Scientific (Ocala, FL, USA), VWR (Radner, PA, USA)

Table 2.5 Apparatuses used for experiments

Apparatus	Company
AccuBlock Digital Dry Bath	Labnet International, Inc.
Beckmann Centrifuge	Beckmann-Coulter
Bio Rocker Model 110A	Denville Scientific Inc
Calipers	VWR
Countess automated cell counter	Invitrogen
C1000™ Thermal Cycler	Bio Rad
CFX96 RealTime System	Bio Rad
Cell culture Hood	VWR
Centrifuge 5702	Eppendorf
Classic Plus pH meter, AB104-S	Mettler Toledo
CO_2 Incubator	SANYO
Dionex Ultimate 3000 HPLC	Thermo Scientific
EppiCentrifuge	Eppendorf
FreeZone 4.5 Plus	Labconco
Incubator	Forma Scientific, Inc.
Mini Mouse Centrifuge	Denville Scientific Inc
MiniSpin® Plus	Eppendorf
My Cycler™ thermal cycler	BIO-RAD
NanoDrop® ND-1000 Spectrophotometer	Thermo Scientific

Scales	Mettler-Toledo
Sonicator FS30	Fisher Scientific
Sorvall St 16 Centrifuge	Thermo Fisher Scientific
Synergy MX	Biotek
SpeedVac Concentrator SAVANT SPD111V	Thermo Fischer Scientific
Vacuum manifold 96 well columns	Sigma-Aldrich
Water bath 2870	Fisher Scientific

Beckman Coulter (Brea, CA, USA), BIO-RAD (Hercules, CA, USA), Biotek (Winoosky, VT, USA) Denville Scientific (South Plainfield, NJ, USA), Eppendorf (Hamburg, Deutschland), Labconco (Kansas City, MO, USA), LI-COR (Lincoln, NE, USA), Mettler Toledo (Columbus, Schweiz), Molecular Devices (Sunnyvale, CA, USA), Nikon (Chiyoda, Tokyo, Japan), Thermo Fisher Scientific (Waltham, MA, USA), SANYO (Moriguchim, Osaka, Japan)

2.2 Methods

2.2.1 Separation, purification and identification of HMO

Pooled HMO (pHMO) used for the experiments were isolated and prepared in the Bode lab with a method described previously (Kunz et al., 1996). Breast milk collected at different times post-partum, was donated by healthy women whom gave birth at term. The milk from more than 50 women was pooled and centrifuged of donor milk to remove the lipid layer. In addition, proteins were removed by precipitation from the aqueous phase with ice-cold ethanol. Ethanol was evaporated using a rotary evaporator and the remainder of the sample was then lyophilized. Lactose and salts were removed by FPLC (2.2.1.1) and samples were analyzed by HPLC (2.2.1.3), where HMO profiles containing less than 2% lactose were pooled and later used for experiments. Individual HMO used in experiments were either generously provided or purchased commercially (Table 2.6).

Table 2.6 Individual HMO used for experiments

Individual HMO	Company
3'Sialyllactose (3'SL)	Jennewein Biotechnologie
6'Sialyllactose (6'SL)	Kyowa
2'Fucosylactose (2'FL)	Jennewein Biotechnologie
Disialyllacto-N-tetraose (DSLNT)	V-labs, INC
Lacto-N-fucopentaose 1 (LNFP-1)	V-labs, INC

2.2.1.1 Fast Protein liquid chromatography

Semi-automated fast protein liquid chromatography (FPLC) was performed to remove salt and lactose from isolated pHMO (2.2.1), via gel filtration and size exclusion chromatography. Bio-Gel P-2 gel, a material made up of fine, porous polyacrylamide beads (prepared by copolymerization of acrylamide and N, N'-methylene-bis-acrylamide) was used as a stationary phase resin. The resin is hydrophilic and free of charge and the narrow distribution of bead diameters (the particles size of the hydrated beads is 45-90 μM) separates the HMO

from lactose and salts by molecular weight discrimination, where large molecules that cannot penetrate into the pores elute first, while smaller structures enter the pores and elute later (Barth et al., 1994; Striegel et al., 2009).

The P-2 gel was allowed to swell in water overnight at 4°C and was then washed 5 times with water and sonicated to remove air bubbles before packing the FPLC column. A glass column, 100 cm long by 16 mm wide, (GE Healthcare Life Sciences, Pittsburg, PA, USA) was used and packed under pressure (1.2 mL/min) with the prepared P-2 material. Once packed, it was connected and set up with the remaining FPLC system (injection loop and valve, pump, UV detector, conductivity monitor, fraction collector, buffer). The pressure within the columns went up to approximately 16 psi (pounds per square inch) and stability in pressure indicated no leakage within the system.

Lyophilized pHMO were each suspended in HPLC grade water and injected onto the FPLC columns. The protocol (volume and flow rate) was defined with the program Biologic DuoFlow (Bio-Rad Laboratories Inc.) and used for the purification of pHMO: 2 mL sample were loaded and injected at a flow rate of 1 mL/min. A total of 177 mL of water was injected on the column at a flow rate of 0.8 mL/min, where within a one-time window, 80 mL (containing the pHMO) are collected from the column, into fractions. The column is washed after collection with 25 mL (flow rate of 0.8 mL/min) to remove remaining lactose and salts.

Individual fractions collected from pooled injections were analyzed by HPLC (2.2.1.3), to determine lactose concentrations in each fraction. Fractions were then manually combined together, after excluding fractions containing >2% lactose. All samples were frozen and lyophilized until completely dry.

2.2.1.2 Ion exchange chromatography

Pooled HMO were separated into non-sialylated (neutral) and sialylated (acidic) fractions by means of quaternary aminoethyl (QAE) anion exchange chromatography. QAE Sephadex® A-25 chloride (20 g) was suspended in 1 M Tris buffer (400 mL) to allow swelling of the material at 4°C overnight. Supernatant was discarded the following day and the QAE material was washed with 400 mL of fresh 1 M Tris buffer. 8 mL of QAE material in 1 M Tris buffer was added and packed to gravity columns, where 50 mL conical tubes were used to collect the flow-through. 2 mM Tris was added to the columns to reach a pH of 7.5-8. 1 g HMO were prepared in 5 mL 2 mM Tris buffer, the pH was adjusted to 7.5-8 with 1 M Tris and HMO were added to each column. For the collection of neutral HMO fractions, 8 times the QAE bed volume (64 mL) of 2 mM Tris was added and the flow through (containing neutral HMO) was collected. Sialylated HMO were eluted by adding 64 mL of 2 mM Tris +

100 mM NaCl buffer to each column. The flow through obtained accounted for the neutral and acidic HMO present in 1 g of pooled HMO. The tubes with the fractioned HMO were frozen and then lyophilized until completely dry.

2.2.1.3 High Performance Liquid Chromatography

10 µL aliquots of individual or combined fractions (2.2.1.1) were pipetted into a 96-well plate and dried down in a SpeedVac system. The reducing end of the oligosaccharides were labeled with fluorescent tag 2-aminobenzamide (2AB) for exactly 2 hours at 65°C. 2AB labeled oligosaccharides were taken up in HPLC buffer B and analyzed by high performance liquid chromatography (HPLC) on an amide-80 column (Tosoh Bioscience, Tokyo, Japan) with a 50 mM ammonium formate/acetonitrile buffer gradient system, where separation was monitored by a fluorescence detector at 360 nm excitation and 425 nm emissions. Peak annotations were based on standard retention times to determine successful removal of lactose, or to identify oligosaccharides in each FPLC fraction collected (Townsend et al., 1996).

Table 2.7 Preparation of 2AB Reagent.
Preparation included weighing out components and was performed under the fume hood. 2AB reagent was stored at -20°C for a maximum of 48 hours.

Chemical	Amount
2-aminobenzamide (anthranilamide)	0.024 g
Sodium cyanoborohydride	0.031 g
Acetic acid, glacial	150 µL
Dimethyl sulfoxide (DMSO)	350 µL

Table 2.8 Preparation of HPLC Buffers

HPLC Buffer A - Chemical	Amount
HPLC water	Adjust to final volume of 1 L
Formic acid	2.6 mL
Ammonium hydroxide	~5 mL to adjust pH to 4.4

HPLC Buffer B - Chemical	Amount
Acetonitrile	80%
Buffer A	20%

2.2.2 Endotoxin reduction in HMO samples

Endotoxin (LPS) was removed from all pHMO and individual HMO used for *in vitro* experiments by Detoxi-Gel Endotoxin Removing columns (Pierce Thermo Scientific, Rockford, IL, USA) according to the manufacturer instructions. The whole procedure was

performed under sterile conditions with the use of pyrogen-free UltraPure distilled water, ethanol 200 proof and sterile filter tips to avoid LPS contamination. After collection of the flow-through, containing HMO, the purified samples were frozen immediately and lyophilized until completely dry. To minimize the amount of LPS, the HMO samples were processed twice on the endotoxin removing columns. To increase the efficiency of the columns, the amount of HMO loaded onto each column was decreased by diluting with UltraPure distilled water to 2 mg pHMO or individual HMO per column. HMO were incubated within the column for 1 h, at room temperature (Gnoth, Kunz, & Rudloff, 2000).

2.2.3 *In vitro* methods

2.2.3.1 Culture of Raw 264.7 cell line

RAW 264.7 macrophages are a murine macrophage cell line established from a tumor induced by Abelson murine leukemia virus (Raschke et al., 1978b). RAW 264.7 macrophages were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Stock vials of cells were stored in liquid nitrogen and later thawed in a 37°C water bath (2.2.3.4). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 0.1% Penicillin-Streptomycin (Pen-Strep) in a humidified incubator with a 5% CO_2 atmosphere at a temperature of 37°C. For routine maintenance in culture, cells were seeded at a confluence of approximately 10%, every 3-4 days using Trypsin EDTA and grown to a confluence of approximately 80-90% prior to experiments. RAW 264.7 macrophages were seeded into 12-well plates (300,000 cells/well) and incubated overnight at 37°C. The next day, culture medium was exchanged with fresh DMEM (supplemented with 10% FBS and 0.1% Pen-Strep) and experiments were conducted the following day.

2.2.3.2 Culture of THP-1 cell line

THP-1 cells are a human monocytic cell line derived from the peripheral blood of a 1-year-old male with acute monocytic leukemia. THP-1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Stock vials of cells were stored in liquid nitrogen and later thawed in a 37°C water bath (2.2.3.4). Cells were cultured in suspension in a 75 cm^2 (T75) culture flask containing 25 mL of THP-1 medium (RPMI supplemented with 10% FBS and 10 mM Hepes), in a humidified incubator with a 5% CO_2 atmosphere at a temperature of 37°C. For routine maintenance in culture, cell suspensions were transferred to 50 mL falcon tubes and spun down for 5 min at 1400 rpm. Cell pellet was resuspended in fresh 10 mL of THP-1 media and cells were counted (2.2.3.5). Suspension containing 1 million cells was transferred to new T75 flask and THP-1 media was added for a final volume of 25 mL. Cells were grown to a confluence of approximately 80-90% and split every 3-4

days. Prior to experiments THP-1 cells split into 12 well plates (1 million cells per well) and were differentiated into macrophages by adding 600 nM phorbol 12-myristate 13-acetate (PMA) for 48 h. Differentiated macrophages attached to plate surface and were used for experiments.

2.2.3.3 Culture of HEK293T cell line

The HEK293T cell line is a highly transfectable derivative of human embryonic kidney 293 cells. This cell line contains the SV40 T-antigen and is therefore competent to replicate vectors carrying the SV40 region of replication, giving high titers when used to produce retroviruses. HEK293T cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Stock vials of cells were stored in liquid nitrogen and later thawed in a 37°C water bath (2.2.3.4). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 0.1% Penicillin-Streptomycin (Pen-Strep) in a humidified incubator with a 5% CO_2 atmosphere at a temperature of 37°C. For routine maintenance in culture, cells were seeded at a confluence of approximately 10% and grown to a confluence of approximately 80-90% prior to experiments. Cells were split every 3-4 days using Trypsin EDTA.

2.2.3.4 Thawing and freezing cells

For longer storage cell lines (Raw 264.7, HEK293T and THP-1) were kept in liquid nitrogen tanks at -197°C. For that purpose, one T25 culture flask (for Raw 264.7 and Hek293T cells) or a T75 flask (for THP-1 cells), at 80-90% confluency, was split into two cryotubes containing: 90% of culture medium and 10% DMSO. Cells were initially frozen at -80°C and after few days transferred into liquid nitrogen tanks.

To rapidly thaw the cells, a cryotube was placed in a 37°C water bath and cells were immediately transferred to 10 mL of pre-warmed media in 15 mL Falcon tube. After being centrifuged at 1200 rpm for 10 min to remove cytotoxic DMSO, the cell pellet was suspended in fresh media and cells were seeded onto a 25 cm^2 or 75 cm^2 culture dish.

2.2.3.5 Counting cells

Counting of cells for experiments was done using a hemocytometer (Hausser Scientific CO, Horsham, PA, USA). The counting chamber is comprised of nine large squares, which are further divided into 16 smaller squares. The re-suspended cells were added to the chamber and counting was performed in 4 exterior larger squares. The concentration of cells was calculated by multiplying the average count from all 4 squares by chamber factor 10^4 (because the volume of one square equals 0.1 µL) to determine cell count per 1 mL.

2.2.3.6 Silenced Target Gene Expression by iRNA (siRNA) and transfection

To target gene silencing of the gene encoding the prostaglandin E_2 receptor 4 (PTGER4) in RAW 264.7 macrophages, Accell siRNA reagents and products were used. Accell products are modified for delivery into difficult-to-transfect cells and require no transfection reagent or viral vector.

Optimal cell densities and growth rate of cells were assessed in Accell Delivery Media prior to carrying out Accell short interfering RNA (siRNA) experiments. Confluent cells were lifted form plates using trypsin and diluted in growth medium to a plating density of 50% confluency into a 12 well plate and incubate cells at 37°C with 5% CO overnight. 100 µM of SMARTpool siRNA solution (containing a mixture of 4 siRNA against mouse PTGER4, or non-targeting siRNA) was prepared in 1X siRNA Buffer and solution was pipetted solution up and down 3-5 times while avoiding introduction of bubbles and placed on an orbital shaker for 90 minutes at 37°C. samples were briefly centrifuged to collect the solution to the bottom of the tubes. 7.5 µL of the 100 µM siRNA was mixed in separate tubes with 750 µL Accell Delivery Media containing less than 0.5% FBS and used immediately at a final concentration 1 µM Accell siRNA per well, in 12 well plate. Growth medium from the cells was removed and 100 µL of the appropriate delivery mix containing Accell siRNA and Delivery Media, were added to each well and incubated at 37°C with 5% CO_2. After 48 h, experiments were then carried out. Cells well were activated with LPS and co-stimulated with 3'SL or phosphate-buffered saline (PBS) for 6 h and relative mRNA expression of PTGER4 and *IL-6* were assessed.

Table 2.9 Pre-designed siRNA products

Accell non-targeting pool (D-001910-10-20) GE Dharmacon
Description: A four component siRNAs designed to have no homology to known mouse genes. Changes in mRNA or protein levels in cells treated with these controls reflect a non-specific baseline cellular response that can be compared to the levels in cells treated with target-specific siRNA

SMARTpool Accell PTGER4 siRNA (E-048700-00-0010) GE Dharmacon
Description: Mus musculus prostaglandin E receptor 4 (subtype EP4) (Ptger4), transcript variant 1 and variant 2, mRNA. NCBI Reference Sequence: NM_001136079.2 and NM_08965.2

2.2.4 Primary cells

2.2.4.1 Bone marrow derived macrophage isolation

C57BL/6J mice were euthanized via CO_2 and cervical dislocation and femur and tibia from both hind legs were each removed at the hip and foot joints. Additionally, femur and tibia were separated from one another at the joints and placed in a dish containing sterile PBS. A 10 mL syringe and 25-G needle was filled with cold sterile PBS (without calcium or magnesium). The lower 4 mm of the tibia bones (where it connected to ankle) were severed and at the opposite end, the needle was used to flush out the bone marrow into a sterile 50 mL conical tube. The process was repeated with the femur bones by cutting the ball portion of femur bones and flushing out the bone marrow, with the needle, at the opposite end. Cells were homogenized with a sterile 10 mL pipette (up and down 3-5 times) and then again with a sterile 10 mL pipette and a 200 µL pipette attached to the end, to thoroughly break up the bone marrow and homogenization. Cells were centrifuged at room temperature, for 10 min at 200 x g. Supernatant was discarded and re-suspended in 30 mL DMEM/F12 (supplemented with 10% FBS, 0.1% Pen-Strep) and 20 ng/mL MCSF was added fresh to the media to stimulate macrophage differentiation. The cell suspension was divided, 10 mL to a 10 cm^2 petri dishes and incubated at 37°C, with 5% CO_2.

2.2.4.2 BMDM culture

Bone marrow derived macrophages were isolated from C57BL/6J mice and cultured on 10 cm^2 petri dishes in an incubator at 37°C, with 5% CO_2 (see 2.2.4.1). Cells were systematically checked for their morphology and culture media containing 20 ng/mL M-CSF (to stimulate macrophage differentiation) was refreshed every other day. On day 5 to 7 post isolation cells were washed with PBS twice and cells well incubated for 5 min with dissociation buffer to lift cells from petri dishes. BMDM were seeded into 24-well plates (450,000 cells/well) and incubated overnight at 37°C in culture media containing 20 ng/mL M-CSF. The following day, cells were used for the experiment.

2.2.5 Activation of RAW 264.7, BMDM and THP-1 cells and exposure to HMO

Raw 264.7, THP-1 and BMDM were seeded as previously described into 12 or 24 well plates (see 2.2.3.1, 2.2.3.2 and 2.2.4.2). On the day of the experiment medium was prepared containing LPS from Escherichia coli 0111:B4 (Sigma-Aldrich, St. Louis, MO, USA) for activation of cells and/or containing endotoxin free pooled or individual HMO. LPS (1 µg/mL LPS in PBS) and HMO (10 µg/µL HMO in PBS) stock solutions (stored at -20°C) were used to prepare the media. Since LPS is a very potent stimulant, LPS stock medium (10 ng/mL LPS in DMEM) was prepared and used for all cells activated with LPS at the day of the

experiment. As a control medium for non-activated cells, PBS stock medium was used. The stock media served as the basis and HMO or PBS were added in the concentration needed. For media without HMO, PBS instead of HMO stock solution was used. To avoid microbial contamination all prepared media were filter sterilized (0.22 µm filter units). Other stimuli were also used to induce inflammatory responses (see Table 2.10), where preparation of media and cell handling followed the procedure explained above.

Table 2.10. Stimuli used to active macrophages

Name	Company	Catalog number
LPS from Escherichia coli 0111:B4	Sigma- Aldrich	L3024
Lipid A, monophosphoryl from Salmonella enterica serotype minnesota Re 595	Sigma- Aldrich	L6895
IFN-β	R&D Systems	8234-MD

2.2.6 Antagonist Assays

BMDM were isolated and cultured as previously described (2.2.4.1, 2.2.4.2). Cells were seeded at 400,000 cells/well in a 24 well plate. Cells were pre-incubated for 30 min at 37°C in BMDM culture media containing different concentrations and combinations of antagonists or agonists (see).

Table 2.11). Cells were then stimulated with LPS (10 ng/mL) and treated with 3’SL (100 µg/mL) as previously described (2.2.5).

Table 2.11. Receptor Agonist and Antagonists

Name	Receptor	Action	Company	Catalog
WRW4	ALX/FPR2	Antagonist	Tocris Bioscience	2262
Boc2 (N-t-Boc-Met-Leu-Phe)	ALX/FPR2	Antagonist	MP Biomedical LLP	152799
CAY10598	EP4	Agonist	Cayman Chemicals	13281
ONOAE3208	EP4	Antagonist	Cayman Chemicals	14522

2.2.7 Gene Expression Analysis

Total RNA from RAW 264.7, BMDM or THP-1 cells was extracted using TRIzol Reagent. In brief, cells were rinsed with 1 mL PBS and incubated with 500 µL TRIzol Reagent for 5 minutes at room temperature, with rocking. Cells were scraped by pipetting thoroughly and transferred into centrifuge tubes. Using 100 µL chloroform and centrifugation (5 minutes at 12,000 g) the top aqueous and RNA containing layer was transferred to a new tube and

mixed with 300 μL 70% ethanol (EtOH). RNA was purified using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer instructions. After RNA was eluted, RNA quantity and quality was measured using NanoDrop Spectrophotometer ND-100.

Total RNA was reverse transcribed to synthesize first-strand complementary DNA (cDNA) using the Bio-Rad iScript cNDA synthesis kit (Bio-Rad, Hercules, CA, USA) in a total volume of 20 μL following the provided guidelines.

Quantitative real-time-polymerase chain reaction (qRT-PCR) was performed to quantify relative mRNA concentrations. Formulations for each cDNA were prepared using 30 μL Kapa SYBR® Fast Universal q-PCR (Kapa Biosystems Inc, Wilmington, MA, USA) and 20 μL of diluted cDNA sample (in UltraPure distilled water) for each reaction. Primer stocks were diluted to a concentration of 10 μM before use and 5 μL forward and 5 μL reverse primer working stocks were added to each well. All samples were run in triplicate. qRT-PCR was carried out on a C1000 Thermal Cycler – CFX96™ Real-Time System. Amplification conditions were as follows: 95°C initial denaturation for 10 minutes; 40 cycles of 95°C for 30 seconds and 55°C for 1 minute followed. Plate reading and detection of the fluorescent signal occurred after each cycle. Relative expression levels of the target genes were calculated based on $2^{-\Delta\Delta Ct}$ by normalizing the gene of interest cycle threshold (Ct) to the TATA-binding protein gene (TBP) as a reference housekeeping gene. Primers were ordered from ValueGene (San Diego, CA, USA). The sequences of forward and reverse primers used for qRT-PCR analyses are listed below.

Table 2.12. Forward and Reverse Primers used for SYBR Green Expression Analysis

Gene-mouse	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
IL-1β	AAATACCTGTGGCCTTGGGC	CTTGGGATCCACACTCTCCAG
IL-6	CCAGAGATACAAAGAAATGATGG	ACTCCAGAAGACCAGAGGAAAT
iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
Ccl2	AGGTCCCTGTCATGCTTCTG	GCTGCTGGTGATCCTCTTGT
ALX/FPR2	CCTTGGCTTTCTTCAACAGC	GCACAGTGGAACTCAAAGCA
EP4	TTCCGCTCGTGGTGCGAGTGTTC	GAGGTGGTGTCTGCTTGGGTCAG
TBP	GAAGCTGCGGTACAATTCCAG	CCCCTTGTACCCTTCACCAAT

Gene-human	Forward primer (5'-3')	Reverse primer (5'-3')
IL-1β	GTGGCAATGAGGATGACTTGTTC	TAGTGGTGGTCGGAGATTCGTA
IL-6	AGCCACTCACCTCTTCAGAAC	GCCTCTTTGCTGCTTTCACAC
TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCTGCCAGTCTGGAC

2.2.8 Multiplex Enzyme-Linked Immunosorbent Assay

2.2.8.1 Sample Preparation

BMDM were plated in 12-well plates (450,000 cells/well) as described in 2.2.4. Prior to analysis, cells were co-incubated for 24 h with 10 ng/mL LPS or PBS and 100 µg/mL 3'SL or PBS as described in 2.2.5. Cell culture supernatants were collected, centrifuged for 4 minutes at 14,500 rpm and the cell-free supernatant was transferred to a new tube. Cytokine production of BMDM was determined using the MSD Multi-spot Assay system and instructions were followed according to the manufacturer's protocol.

2.2.8.2 Pro-inflammatory assay

BMDM cell supernatants were added (in duplicate) to a pro-inflammatory 96-well panel measuring 10 murine cytokines (*IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, CXCL1, IL-10, IL-12p70* and *TNF-α*) important in inflammation response and immune system regulation. The assay measures biomarkers that are implicated in a number of disorders, including rheumatoid arthritis, atherosclerosis, obesity and diabetes. The assay was a sandwich immunoassay. The plate provided was pre-coated with capture antibodies on independent and well-defined spots. The sample was diluted (1:2 and 1:10) in a solution containing detection antibodies conjugated with electrochemiluminescent (ECL) labels. The diluted samples were added to the 96-well plate and incubated overnight. Analytes in the sample bound to capture antibodies immobilized on the working electrode surface and the recruitment of the detection antibodies by the bound analytes completed the sandwich. A buffer provided in the kit, (creating the appropriate chemical environment for electrochemiluminescence) was added to the 96-well plate and the plate was loaded into an MSD instrument where a voltage was applied to the plate electrodes, causing the captured labels to emit light. The instrument measured the intensity of emitted light (proportional to the amount of analyte present in the sample) and provided a quantitative measure of each analyte in the sample. Analyte concentrations were determined from the ECL signals by back-fitting to the calibration curve. The calculations to establish calibration curves and determine concentrations were carried out using the MSD Discovery Workbench software, version 4.0 (Meso Scale Diagnostics, Rockville, MD, USA).

2.2.9 Genetic Analysis and Validation of RNA Sequencing

Transcriptomes of quiescent BMDM (PBS) were compared to BMDM incubated with either 3'SL or LPS alone and LPS and 3'SL together. RNA was extracted from cells, converted to cDNA and the sizing library construction and RNA sequencing was performed by the University of California San Diego (UCSD) Institute for Genomic Medicine and analyzed by a

bio-informatics laboratory of Dr. Nathan Lewis, at UCSD. RNA sequencing (RNA-seq) data was analyzed the by clustering genes by their functions and common activated or repressed transcription factors. Transcripts were mapped to the mouse genome and differently expressed genes were quantified by counting the number of sequence reads per gene and comparing the counts between samples. Different comparisons were made (ex: PBS vs. 3'SL; PBS vs. LPS; PBS vs. LPS+3'SL; LPS vs. LPS+3'SL). Fisher's exact test was performed to identify transcripts with significantly differential expression between samples. To determine the expression change direction, "greater" or "less" parameters in the one-tailed Fisher's exact test were used to find the up-regulated transcripts or down-regulated transcripts, respectively (Chen et al., 2016; Liu et al., 2014; Robinson & Oshlack, 2010). To evaluate statistical significance of the results the adjusted p-value for each test was determined. Taking into account the false discovery rate (FDR) is necessary for measuring thousands of variables, such as gene expression levels, from the small sample set. Genes were considered differentially expressed if the adjusted p-value was less than 0.05, implying that 5% of the tests found to be statistically significant by raw p-value ($p<0.10$ for each test) will be false positives (Jun Li et al., 2012).Genes with an adjusted p-value great than 0.05 and a raw p-value greater than 0.10 were excluded.

Differential gene expressions between experimental groups were determined by a log fold change. A positive $\log_2$-fold-change (logFC) indicated that a gene was up-regulated whereas a negative logFC indicated that gene was down-regulated. Genes significantly differentially expressed 1.5-fold or greater were used for analysis. Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis Jr et al., 2003) and Ingenuity Pathway Analysis (IPA) (Krämer et al., 2014) programs were used to annotate genes by their biological process, molecular function and cellular component. IPA was used to predict upstream regulators of the differentially expressed genes. The distinct up- and down- regulation pattern of the expressed genes are taken into account in combination with relationships previously reported in the literature, to predict whether such regulators are activated or inhibited. Any gene or small molecule observed to affect gene expression in some direct or indirect way were reported as an upstream regulator. The likely upstream regulators and networks constructed by the software were sorted by statistical significance. Fisher's exact test (FET) p-value provides and enrichment score that measures overlap of observed and predicted regulated gene sets ($p<0.05$), without taking into account gene direction (Robinson & Oshlack, 2010). In addition, the activation Z-score makes predictions about potential regulators by using information in the knowledge database about the direction of gene regulation (Cheadle et al., 2003). A Z-score greater than 2, or less than minus 2 projects that a transcript regulator is significantly activated or inhibited, respectively. Taken together, both scores serve as a significant measure and a predictor for the activation state of the regulator.

HOMER (Hypergeometric Optimization of Motif EnRichment) was used as an online Motif discovery tool to identify transcription factor binding sites in the promoters of the differential expressed genes (Barash et al., 2001). The promoter regions of the differentially expressed genes were scanned to identify common transcription factor binding sites and identify the dominant ligand-receptor pathway activated and/or inhibited by 3’SL.

2.2.10 Conversion of macrophage to foam cells

2.2.10.1 Foam cell Assay

Foam cell conversion is determined by the uptake of cholesterol from modified LDL, macrophage lipid biogenesis (cholesterol esterification) and cholesterol efflux. BMDM were pated (350,000 cells per well) in a 24-well plate as previously described 2.2.4.2 and stimulated with culture DMEM containing either oxidized LDL or aggregate LDL (50 µg/mL) and additionally co-incubated with either 3’SL (100 µg/mL) as a treatment, or PBS as a control, for 24 h at 37°C and 5% CO_2. After 24 h co-incubation, cells were washed with PBS three times and 200 µL of ice-cold 4% paraformaldehyde (PFA) was added to each well for 2 min on ice. Cells were rinsed again three times with PBS and 200 µL of ice cold 100% EtOH was added to each well and incubated for 10 min at 4°C, with mild shaking. Ethanol supernatants were carefully transferred to micro-centrifuge tubes kept on ice and later used for cholesterol detection. Cells were washed once with PBS and incubated with 100 µL of a lysing solution coating 0.1 M NaOH and 1 mg/mL sodiumdodecylsulphate (SDS), for 30 min with shaking, at 4°C. Cells were then scraped and transferred to microcentrifuge tubes and centrifuged for 5 min at 500 g. 10 µL of supernatant from cell lysate was used to determine protein concentration for each sample using a bicinchoninic acid (BCA) protein assay kit (Pierce Thermo Scientific, Rockford, IL, USA.)

2.2.10.2 Detection of cholesterol uptake

The Amplex® Red Cholesterol Assay Kit was used to detect both free cholesterol and cholesteryl esters in BMDM supernatant. The assay provides a simple fluorometric method for the sensitive quantitation of cholesterol using a fluorescence microplate reader (Biotek, VT, USA). The assay is based on an enzyme-coupled reaction able to detect both free cholesterol and cholesteryl esters. Cholesteryl esters are hydrolyzed by cholesterol esterase into cholesterol and then oxidized by cholesterol oxidase, yielding H_2O_2 and the corresponding ketone product. H_2O_2 is then detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red reagent). Amplex® Red reagent reacts with H_2O_2, in the presence of horseradish peroxidase (HRP), to produce highly fluorescent resorufin. The

assay was performed following the manufacter's protocol and fluorescence was detected at 571 nm and 585 nm, the absorption and fluorescence emission of resorufin, respectively.

Cholesterol standard curve was prepared by diluting the 2 mg/mL (5.17 mM) cholesterol reference standard (Component H provided in the kit) into 1X reaction buffer (provided in the kit) to produce cholesterol concentrations of 0, 0.5, 1, 2, 4 and 8 µg/mL (0 to ~20 µM). 40 µL of the cholesterol containing samples were diluted in 160 µL 1X reaction buffer. 50 µL of the diluted samples and controls were pipetted in duplicates into separate wells of a 96-well plate. Two different working solutions were prepared, both containing 300 µM Amplex® Red reagent, 2 U/mL HRP and 2 U/mL cholesterol oxidase. One of the working solutions contained 0.2 U/mL cholesterol esterase, whereas the ladder did not. 50 µL of the Amplex® Red reagent/HRP/cholesterol oxidase/ with (or without) cholesterol esterase working solution was added to each well containing duplicates of the samples and controls. The reaction was incubated for 30 minutes at 37°C, protected from light. Cholesterol concentrations were measured by fluorescence in microplate reader, using excitation in the range of 530–560 nm and emission detection at ~590 nm. For each point, background fluorescence was corrected by subtracting the values derived from the no-cholesterol control. Samples incubated with cholesterol esterase generated a quantitative value for total cholesterol, whereas samples incubated without the enzyme generated a quantitative value for free cholesterol. For each sample, quantification of cholestryl esters was determined by the subtracting value for free cholesterol from that of total cholesterol. The concentration of cholestryl esters per sample was normalized to the total protein concentration (determined by BCA assay) in each sample.

2.3 *In vivo* methods

2.3.1 Pharmacokinetics of 3'SL in mice

Male mice of C57BL/6J background, around 6-8 weeks of age, were used for pharmacokinetic studies of 3'SL. Different concentrations of 3'SL were either injected intravenously (i.v.), intraperitonealy (i.p.), or by oral gavage. Studies were always performed in triplicates (n=3). Blood was collected by tail vein at different time points in heparinized blood collection tubes and kept on ice. Samples were then centrifuged for 5 min at 2000 rcf and plasma was collected and stored at -20 °C, until used for analysis. In order to determine systemic concentrations of 3'SL in mice, samples were analyzed by HPLC. 10 µL of plasma was worked up of C18 and carbograph packed columns to get rid of all components other than oligosaccharides. Samples were fluorescently tagged at the reducing end with 2-aminobenzamide and worked up over silica packed columns to get rid of non-bound 2AB. Samples were analyzed by HPLC (described 2.2.1.3). 3'SL concentrations were normalized

to known LNFP-1 concentrations (0.5 µg), which was added to each 10 µL plasma sample before HPLC work up.

2.3.2 CAIA Rheumatoid Arthritis mouse model

Collagen antibody-induced arthritis (CAIA) is a mouse model of rheumatoid arthritis, where arthritis is stimulated by the administration of a cocktail of monoclonal antibodies targeting collagen type II and followed by the administration of endotoxin (LPS). Balb/c mice (n=25) were injected i.v. on day 0 with 1.5 mg of Arthrogen-CIA monoclonal antibody cocktail containing A2-10 (IgG2a), F10-21 (IgG2a), D8-6 (IgG2a), D1-2G (IgG2b) and D2-112 (IgG2b) (Chondrex, Inc.) and received an i.p. injection on day 3 of 25 µg of LPS from E. Coli 0111:B4 (Chondrex, Inc.). At time of LPS administration, mice received either an initial oral gavage treatment of 90mg 3’SL (n=12), or water as control (n=13). Treatment was administered every 8 hours thereafter, until the study was terminated. Arthritis was assessed for the presence of erythema or edema in metatarsal/ metacarpal, metatarsophalangeal/ metacarpophalangeal, tarsal/ carpal and phalangeal joints in each paw.

Arthritis in each limb was determined in a blinded manner once per day, where a score of 1 was given for each limb affected, resulting in a maximum score of 4 per mouse. The severity of arthritis was measured on a clinical scale of 0-4, with a maximum total score of 16 for each mouse (Table 2.13). Disease incidence was scored by assigning a score of 2 cumulatively to each anatomic joint that showed evidence of arthritis and a score of 1 was assigned to each digit. By combining the scores of all four limbs, a maximum score of 28 was possible for each mouse.

In addition, both hind limbs were measured before and during the study, using sing a digital Vernier caliper (VWR, West Chester, PA, USA) placed across the ankle joint at the widest point to establish values for ankle thickness (measurements in mm). The study was terminated on day 14 as previously described (Cantley et al., 2011) and mice were humanely euthanized by CO_2 followed by cervical dislocation. Hind paws were collected and fixed in 10% formalin in PBS at pH 7.4 and decalcified in water containing 30% formic acid and 10% formaldehyde for 24 h. Tissues Sections cut 4–7-mm-thick were prepared from the tissue blocks embedded in paraffin and stained with hematoxylin and eosin (H&E) staining was used to determine of inflammation. Toluidine blue stain was used to assess cartilage and bone damage according to published criteria (HistoTox, Boulder CO). All slides for histopathology were observed under light microscopy at a magnification of ×50 and scoring was performed blindly using a previously validated scoring system (Guma et al., 2009; Tak et al., 1997). Scoring of inflammation was based on synovial infiltration with inflammatory cells. Joints were given scores of 0–4 for bone erosion and cartilage depletion was identified by

presence of diminished Safranin O staining of the matrix and was also scored on a scale of 0–4 (Table 2.14). Extensive training and practice are critical to repeatable scoring of CAIA and proper histologic analyses and was therefore performed in a blinded manner by personnel in the lab of Dr. Maripat Corr, at the University of California San Diego.

Table 2.13. Score 16 - Clinical Scoring in RA Mouse Models

Score	Degree of Inflammation
0	No evidence of erythema & swelling
1	Erythema & mild swelling confined to the tarsals or ankle joint
2	Erythema & mild swelling extending from the ankle to the tarsals
3	Erythema & moderate swelling extending from ankle to metatarsal joints
4	Erythema & severe swelling encompass ankle, foot & digits, or ankylosis of limb

Table 2.14 Histological scoring of Arthritic Joints in RA Mouse Models

Score	Degree of Inflammation
0	Normal, <5% inflammatory cells
1	Mild, 5-20 % inflammatory cells
2	Moderate, 20-50% inflammatory cells
3	Severe, with massive immune cell infiltrations (>50%)

Score	Degree of Bone Erosion
0	Normal
1	Minimal (small areas of erosion, not readily apparent on low magnification)
2	Mild (more numerous areas of erosion, not readily apparent on low magnification, in trabecular corcorticol bone
3	Moderate (obvious erosion of trabecular and cortical bone, without full-thickness cortex defects; loss of some trabeculae; lesions apparent on low magnification)
4	Marked (full-thickness defects in the cortical bone and marked trabecular bone loss

Score	Degree of Cartilage Depletion
0	No cartilage destruction (full Safranin-O staining)
1	Localize cartilage erosions
2	More extended cartilage erosions
3	Severe cartilage erosions
4	Depletion of entire cartilage

2.4 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) or standard deviation (SD). Statistical analyses were performed using GraphPad Prism (version 6.0, GraphPad Software Inc, La Jolla, CA, USA). Statistical significance with a criterion of $p < 0.05$, was evaluated

with the unpaired student's T test when comparing two groups with a normal Gaussian distribution of the samples. Otherwise the Mann-Whitney test was used to compare the median values between groups. For experiments with more than two experimental groups, statistical significance was determined by ANOVA test for multiple comparisons. Dunnett's post hoc tests were used for multiple comparisons to a control group and Bonferonni post hoc tests for multiple pair-wise comparisons p-values are indicated by asterisk, where * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; and N.S. = not significant.

3 Results

3.1 Effects of HMO on LPS-induced activation of various macrophage models, *in vitro*

Numerous studies have explored the modulatory properties of HMO on the immune system and shown that HMO have anti-inflammatory effects on different immune cell populations (described in 1.2). In addition, previous work in the lab of Dr. Lars Bode (Szyszka, 2014) provided the first evidence of HMO having anti-inflammatory effects on macrophages. Pooled HMO (pHMO) reduced the gene expression of LPS-induced inflammatory cytokines *IL-1β* and *IL-6* in the macrophage cell line RAW 264.7, in a dose-dependent manner. Neutral and acidic HMO fractionated from pHMO were also tested to help define the specific HMO causing the altered macrophage response to LPS. In this study, the immunomodulatory effects and potential of HMO to reduce chronic low-grade inflammation were further explored using the mouse macrophage cell line RAW 264.7, murine bone marrow derived macrophages, and the THP-1 human monocytic cell line. The intention was to reveal the active HMO structure and to explore the involved underlying molecular mechanisms responsible for the inhibition of LPS-mediated macrophage activation.

All *in vitro* macrophage models were treated with the TLR4 agonist LPS to activate pro-inflammatory signaling cascades and the release of subsequent pro-inflammatory mediators. Low concentrations of LPS (10 ng/mL) were used to mimic the induction of chronic low-grade inflammatory conditions.

Expression and production of inflammatory mediators in LPS-activated macrophages were measured by means of qRT-PCR and ELISA. Fold changes in gene expression and protein concentrations are depicted compared to LPS-stimulated cells (10 ng/mL) without HMO exposure. Statistical significance was determined by normalizing each sample ΔΔCT values to the housekeeping gene, TBP ΔΔCT values.

3.1.1 Effects of pooled neutral and acidic HMO sub-fractions on pro-inflammatory cytokine expression

The first objective of the study was to identify a specific HMO structure responsible for the anti-inflammatory effects observed in LPS-stimulated macrophages exposed to pHMO. A multi-step approach was taken, where pHMO were separated by charge (2.2.1.2) into non-sialylated (neutral) and sialylated (acidic) fractions. They were further separated into neutral subfractions (N1 - N10) and acidic subfractions (A1 - A10), to investigate the structure-specific effects of pHMO. Analysis of HPLC chromatograms (not shown) helped to identify

the most abundant HMO in each fraction (Table 3.1). Chromatograms revealed that samples (N1, N2, N3, A1, A2, A9, A10) contained minimal HMO and therefore fractions N4 through N10 and A3 through A8 were used for experiments on RAW 264.7 macrophages. Since the process separates pHMO by molecular size, the first fraction contained larger molecules, while smaller HMO appeared in later fractions (Barth et al., 1994; Striegel, 2009) and successful separation was confirmed via HPLC chromatograms (not shown), as peaks shifted from longer to shorter retention times.

Table 3.1 HMO detected in neutral and acidic sub-fractions and tested in RAW 264.7 murine macrophages
Cells were exposed to seven neutral fractions and six acidic fractions, and analyzed for relative expression of *IL-1β* and *IL-6*. In this table only the HMO identified by the highest and most distinct peaks in chromatograms are shown. N=neutral fraction, A=acidic fraction.

Fraction	Detected HMO
N 4	Molecules with high molecular size
N 5	Molecules with high molecular size but shift to smaller size (e.g. FLNH)
N 6	LNDFH I or II, LNFP I
N 7	LNFP I, DFLNT, LNDFH I or II, LNT
N 8	LNT, LNnT, DFLNT, LNFP I
N 9	LNT, (3’SL, 2’FL, DFLNT)
N 10	2’FL, 3’SL
A 3	Molecules with high molecular size
A 4	DSLNT
A 5	DSLNT, LST a, b, c
A 6	6’SL, LST c
A 7	6’SL, 3’SL
A 8	6’SL, 3’SL

RAW 264.7 macrophages were incubated with endotoxin-free neutral or acidic sub-fractions of pHMO for 6 hours while being exposed to LPS simultaneously. The concentration of the separated neutral and acidic pHMO sub-fractions was tested relative to 500 µg/mL pHMO, and included an estimated 50% loss during the fractionation processing. Previous experiments in the lab indicated that treatment with pHMO was most effective in reducing relative expression of *IL-1β* and *IL-6.* The same cytokines were therefore tested in RAW 264.7 macrophages treated with pHMO sub-fractions (Figure 3.1 A and B, respectively). qRT-PCR analysis revealed a reduction in gene expression of *IL-1β* and *IL-6* by several fractions (white bars) compared to LPS-stimulated macrophages (red bars). Fractions N10 and N8 were the exception and increased *IL-1β* and *IL-6* expression, respectively. Among the acidic fractions, A4, A5, A7, and A8 had the strongest inhibitory effect on *IL-1β* and *IL-6*.

The HPLC chromatograms suggested that DSLNT was the most abundant HMO in A4 and was also present in fraction A5, along with LST a, b and c. Both 6’SL and 3’SL were found in fraction A7 but were especially abundant in fraction A8. Among the neutral fractions, acidic HMO such as 3’SL, among others, could be detected in fractions N9 and N10. Fractions N6 and N7 showed the strongest inhibitory effects and contained LNDFH I or II and LNFP I, DFLNT and LNT, respectively.

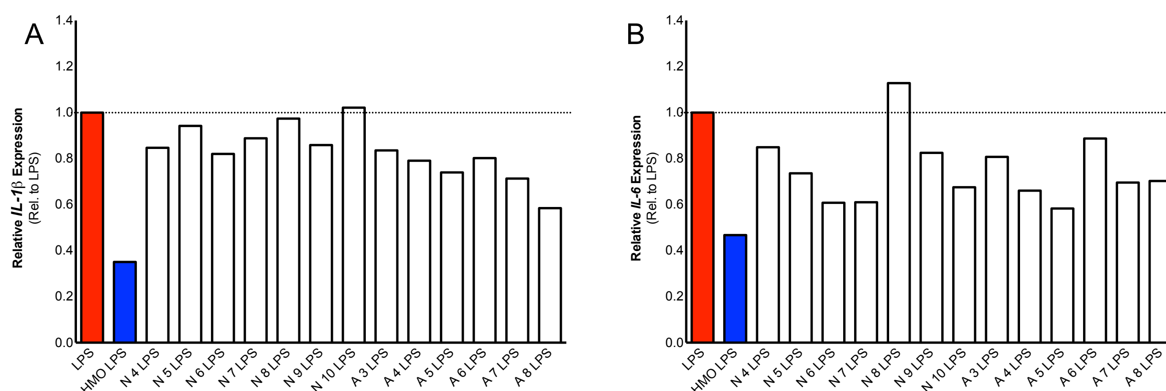


Figure 3.1 Several neutral and acidic sub-fractions of pHMO decrease relative expression of *IL-1β* (A) and *IL-6* (B) in LPS-activated RAW 264.7 macrophages.
Cells were activated with 10 ng/mL LPS for 6h and simultaneously exposed to one of 7 neutral fractions (N4 - N10) or one of 6 acidic fractions (A3 - A8), containing specific HMO in a concentration relative to 500 µg/mL pooled HMO. Total RNA was isolated and qRT-PCR analysis conducted to measure mRNA levels of *IL-1β* (A) and *IL-6* (B). Data are shown as the fold induction of gene expression relative to LPS (10 ng/mL) treatment (normalized to the housekeeping gene TBP). n=1, N=neutral fraction, A=acidic fraction.

3.1.2 Specific HMO attenuate gene expression of inflammatory cytokines in the murine cell line, RAW 264.7 macrophages

The HMO sub-fractions each contained several HMO, and therefore an inhibitory effect on cytokine expression was observed in multiple sub-fractions when exposed to RAW 264.7 macrophages. Limited by commercial availability, only the individual HMO, DSLNT, LNFP-1, 3'SL and 6'SL, which were present in the most effective sub-fractions, were tested. The fraction N10 did not alter *IL-6* expression but instead increased *IL-1β*. The HMO 2’FL was therefore used as a negative control (in addition to LPS with no treatment), and pHMO was used as a positive control. Each individual HMO makes up approximately 20% or less of the total HMO in pHMO, and therefore physiologically relevant concentrations of 100 µg/mL (20% in proportion to 500 µg/mL pHMO) were used for experiments. Individual HMO, verified to be endotoxin-free, were studied and exposed to RAW 264.7 macrophages during the 6

hour LPS-activation period. Analysis showed that pHMO, 3'SL, and 6'SL reduced relative expression of *IL-6* (Figure 3.2 A) and *IL-1β* (Figure 3.2 B) by 50% or more when compared to LPS alone (red bars). LNFP-1 reduced expression of pro-inflammatory cytokines by less than 50% and the reduction was significant for *IL-1β* expression ($p < 0.05$), but not for *IL-6*. In comparison, 2'FL and DSLNT did not alter relative expression of the cytokines *IL-6* and *IL-1β* in LPS-activated macrophages.

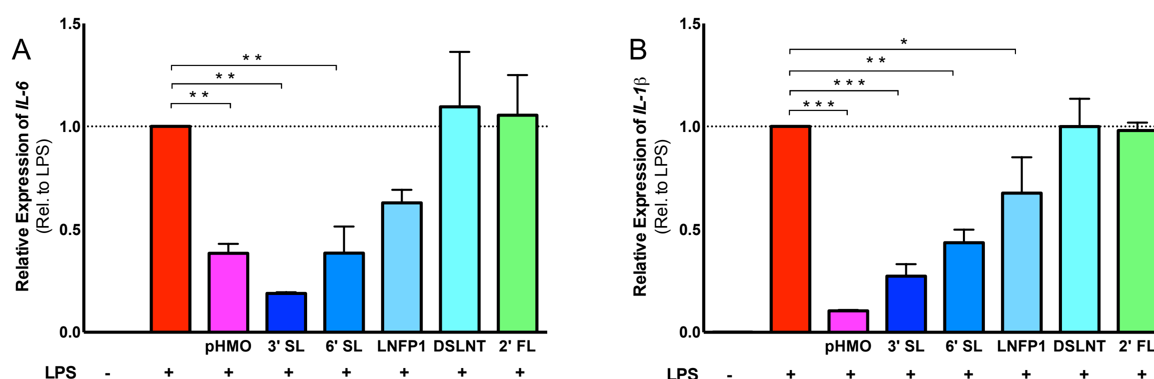


Figure 3.2 Pooled HMO and specific individual HMO reduce relative *IL-6* (A) and *IL-1β* mRNA expression (B) in LPS-activated Raw 264.7 macrophages. Raw 264.7 macrophages were simultaneously exposed to LPS (10 ng/mL) and either PBS (as intervention control), pHMO (500 µg/mL), 3'SL, 6'SL, LNFP1, DSLNT, or 2'FL (each at 100 µg/mL) for 6 h. Cells cultured in DMEM without LPS and without any HMO served as negative activation control. Data shown are the fold induction of gene expression relative to LPS (10 ng/mL) without HMO treatment (normalized to the housekeeping gene TBP). One-way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (* $p<0.05$; ** $p<0.01$; *** $p<0.001$); Bars represent mean ± standard deviation; n=2

These results indicate that the observed effects were highly dependent on structure specificity and that the inhibition of cytokine expression in LPS-activated macrophages was not dependent on the similarities in fucosylation or sialylation between individual HMO (Figure 3.3).

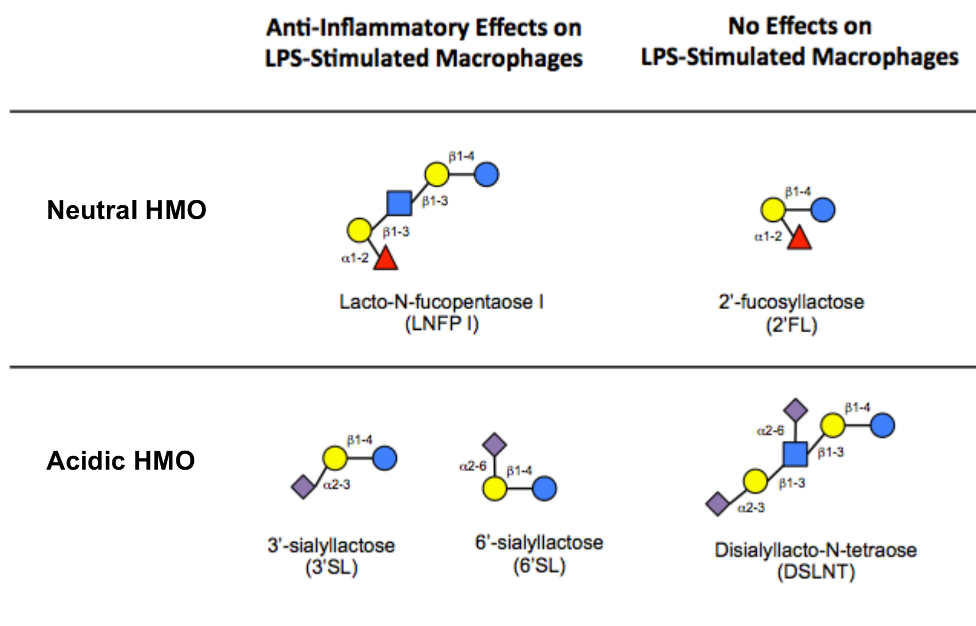


Figure 3.3 Summary and comparison of HMO structures tested in RAW 264.7 macrophages.
Neutral HMO LNFP I and 2'FL and acidic HMO 3'SL, 6'SL, DSLNT have different effects on expression of pro-inflammatory cytokines in macrophages.

3.1.3 HMO attenuate gene expression of inflammatory cytokines in bone marrow derived macrophages

The second objective of the study was addressed by repeating experiments in various macrophage models. In order to generate data with a more relevant representation of living systems, experiments were performed using primary cells, which are known to more closely mimic the physiological state of cells *in vivo*. Bone marrow-derived macrophages (BMDM) are murine primary cells derived from bone marrow and differentiated into macrophages in the presence of macrophage colony-stimulating factor (M-CSF). BMDM were stimulated with LPS for 6 hours and simultaneously treated with pHMO and individual HMO: 2'FL, DSLNT, LNFP-1, 3'SL, and 6'SL. Similar to observations in RAW 264.7 macrophages, pHMO and 3'SL significantly inhibited *IL-6* (Figure 3.4 A) and *IL-1β* (Figure 3.4 B) by more than 50% ($p<0.05$). The reduction in relative cytokine expression by 6'SL was not as prominent, resulting in less than 50% reductions for *IL-6* and only 20% for *IL-1β*. Although there was an apparent trend, results were not significant. Treatment with LNFP-1, which decreased cytokine expression in RAW 264.7 macrophages, was no longer effective in BMDM and was similar to DSLNT and 2'FL.

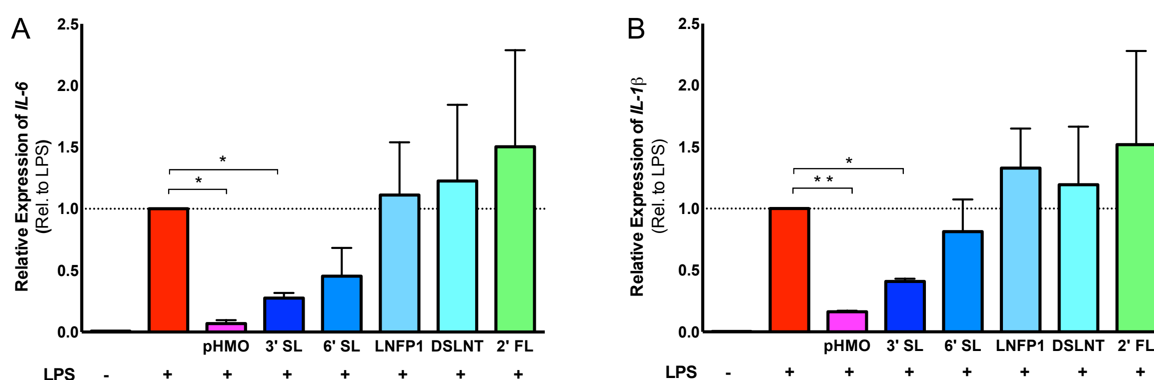


Figure 3.4 Pooled HMO and specific individual HMO reduce relative *IL-6* (A), *IL-1β* mRNA expression (B), in LPS-activated murine bone marrow derived macrophages. BMDM cells were simultaneously exposed to LPS (10 ng/mL) and either PBS (as intervention control), pHMO (500 µg/mL), 3'SL, 6'SL, LNFP1, DSLNT, or 2'FL (each at 100 µg/mL) for 6 hrs. Cells cultured in DMEM without LPS and without any HMOs served as negative activation control. Data shown are the fold induction of gene expression relative to LPS (10 ng/mL) without HMO treatment (normalized to the housekeeping gene TBP). One-way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (* $p<0.05$; ** $p<0.01$); Bars represent mean ± standard deviation; n=2

3.1.4 Determination of 3'sialyllactose dose-dependent response in bone marrow derived macrophages

To further investigate the anti-inflammatory properties of individual HMO on macrophages, the potency of both 3'SL and 6'SL was examined by investigating the dose-dependent responses in LPS-activated BMDM. Macrophages were exposed to concentrations of 3'SL or 6'SL (ranging from 1 mg/mL to 1 ng/mL) that exceeded or were lower than physiologically available in the systemic circulation of infants. This was done to investigate whether 3'SL or 6'SL further enhances or reverses the anti-inflammatory response at high concentrations, and if the ability to attenuate pro-inflammatory mediators continues to be apparent at low concentrations.

LPS-stimulated BMDM were treated for 6 hours with concentrations of 3'SL at 0 ng/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL and 1 mg/mL; or treated with 6'SL concentrations of 0, 10 ng/mL, 100 ng/mL, 1 µg/mL, 10 µg/mL and 100 µg/mL. The inhibitory effects of 6'SL (in green) on *IL-6* (Figure 3.5 A) were only observed at concentrations of 10 and 100 µg/mL and were dose dependent. Concentrations of 100 ng/mL 6'SL reduced *IL-1β* (Figure 3.5 B) gene expression by approximately 40%. No further reduction was observed at higher concentrations and results were not statistically significant. Relative gene expression levels of pro-inflammatory cytokines showed tendencies towards a decrease in expression with increasing doses of 3'SL (in blue). The higher the 3'SL dose, the

lower the level of *IL-6* (Figure 3.5 A) and *IL-1β* gene expression (Figure 3.5 B), when compared to LPS-activated cells without 3'SL treatment. The observed pattern of dose dependence is characterized by a sigmoidal curve. Concentrations of 1 and 10 µg/mL 3'SL showed a trend towards a decrease in *IL-6* expression by approximately 25%. 100 µg/mL 3'SL significantly inhibited expression by approximately 75% ($p<0.05$) and 1 mg/mL by more than 90% ($p<0.05$). *IL-1β* expression was reduced by 1 ng/mL, 10 ng/mL, 100 ng/mL, 1 µg/mL and 10 µg/mL 3'SL, but reduction at these concentrations were not significant. However, higher concentrations of 3'SL at 100 µg/mL and 1 mg/mL significantly inhibited *IL-1β* expression by approximately 75% ($p<0.05$) and at least 90% ($p<0.05$), respectively. These results reveal that 3'SL was more effective than 6'SL in reducing both *IL-6* and *IL-1β* gene expression in LPS-activated BMDM and that the effect of 3'SL was dose-dependent. The IC_{50} value for 3'SL was determined to be 36 µg/mL for *IL-6* and 43 µg/mL for *IL-1β* expression. Experiments presented showed a profound and consistent response with 100 µg/mL 3'SL. To maintain physiological relevance all of the following *in vitro* experiments were conducted only using this concentration.

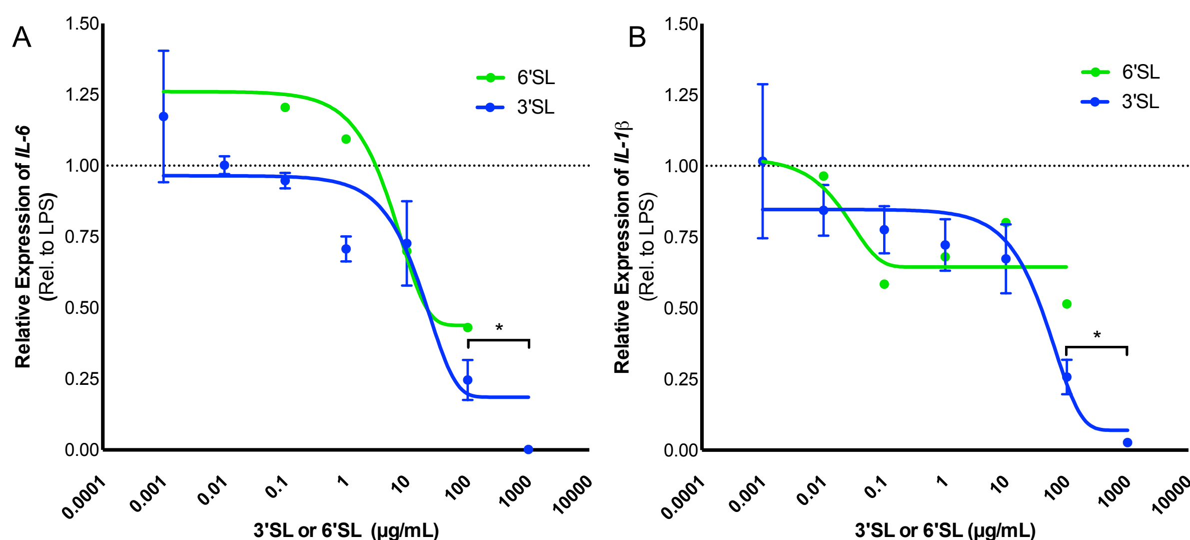


Figure 3.5 3'sialyllactose and 6'sialyllactose reduce relative *IL-6* (A) and *IL-1β* mRNA expression (B), in a dose responsive manner, in LPS-activated murine bone marrow derived macrophages.
BMDM were simultaneously exposed to LPS (10 ng/mL) and 3'SL (10-fold serial dilutions from 1 ng/mL to 1 mg/mL) for 6 h. Cells cultured in DMEM without LPS and without any HMO served as negative activation control. Data shown are the fold induction of gene expression relative to LPS (10 ng/mL) without 3'SL or 6'SL treatment (normalized to the housekeeping gene TBP). Multiple T-tests were used to show the statistical significance between treatments (* $p<0.05$); Bars represent mean ± standard deviation; n=3-4

3.1.5 3’sialyllactose attenuates gene expression of inflammatory cytokines in human monocytic cell line, THP-1 cells

A human macrophage cell line was used to determine if the observed effects by 3'SL in a mouse macrophage cell line and primary cells were also relevant to human cells. Experiments were performed using human monocytic THP-1 cell line, which grew in suspension and were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA). Once differentiated, the cells became adherent and similarly to macrophages obtained from freshly isolated human monocytes, they expressed markers such as *IL-6* and *IL-1β* in the presence of LPS. Differentiated cells were exposed to both LPS and 3'SL for 6 hours, and relative gene expression of *IL-6* and *IL-1β* was assessed. As observed in both *in vitro* mouse macrophage models, 3’SL (blue) also significantly reduced *IL-6* (Figure 3.6 A) and *IL-1β* in the *in vitro* human macrophage model by 50% or more (p<0.01) (Figure 3.6 B) when compared to cells with LPS alone (red).

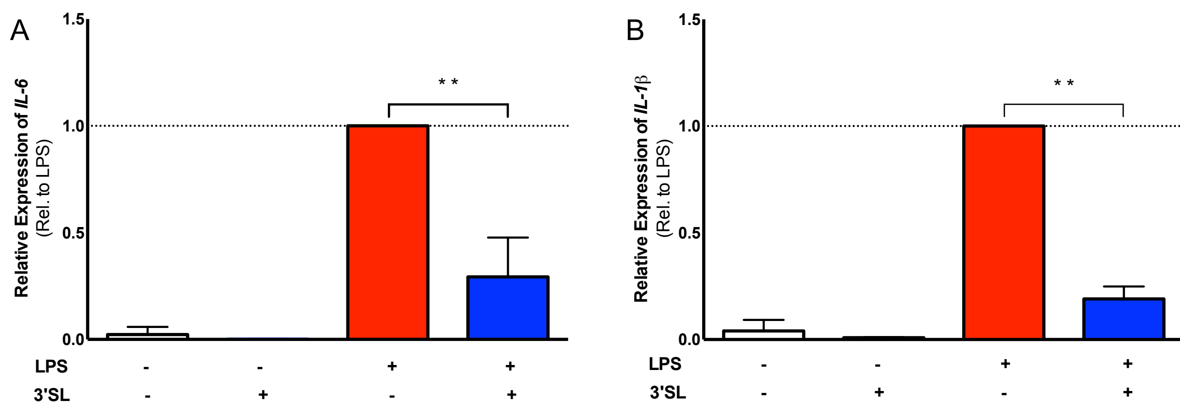


Figure 3.6 3’sialyllactose reduces relative *IL-6* (A) and *IL-1β* mRNA expression (B) in LPS-activated human monocytic cell line.
THP-1 cells were simultaneously exposed to LPS (10 ng/mL) and either PBS (as intervention control), or 3’SL (100 µg/mL) for 6 h. Cells cultured in DMEM without LPS and without any 3’SL served as negative activation control. Data shown are the fold induction of gene expression relative to LPS (10 ng/mL) without 3’SL treatment (normalized to the housekeeping gene TBP). One way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (** p<0.01); Bars represent mean ± standard deviation; n=3

3.1.6 3'sialyllactose reduces relative gene expression of additional inflammatory markers in bone marrow derived macrophages

Exposure to LPS polarizes macrophages towards the M1 phenotype. In addition to inducing the secretion of large amounts of pro-inflammatory cytokines, M1 macrophages have also been shown to activate inducible nitric oxide synthase (*iNOS*) to produce NO, an important pro-inflammatory cytotoxic mediator (MacMicking et al., 1997, Arnold et al., 2014). *iNOS* is a signature molecule for M1 macrophages and is implicated in initiating and sustaining inflammation. In addition, the monocyte chemoattractant protein-1 (MCP-1 or also known as *CCL2*) is one of the key chemokines, induced by LPS and regulates migration and infiltration of monocytes/macrophages and is involved in various diseases. Migration of monocytes from the blood stream across the vascular endothelium is required for routine immunological surveillance of tissues and inflammatory response (Ingersoll et al., 2011).

The second objective of the study was further addressed in the following experiments by determining if the mechanisms of 3'SL include effects that go beyond inhibiting *IL-6* and *IL-1β* cytokine expression. LPS-stimulated BMDM were treated simultaneously with either PBS (white), 3'SL (blue) or pHMO (pink) for 6 hours. The effects on expression of *CCL2* (Figure 3.7 A) and *iNOS* (Figure 3.7 B) were investigated and compared to cells treated with LPS alone (red). Results indicate that the relative gene expression of *CCL2* was significantly reduced by more than 50% ($p<0.01$) by both pHMO and 3'SL (blue and pink bars). In addition, 3'SL also significantly reduced relative *iNOS* gene expression by 50% ($p<0.01$). Incubation with pHMO almost completely attenuated gene expression, reducing *iNOS* by 90% ($p<0.001$).

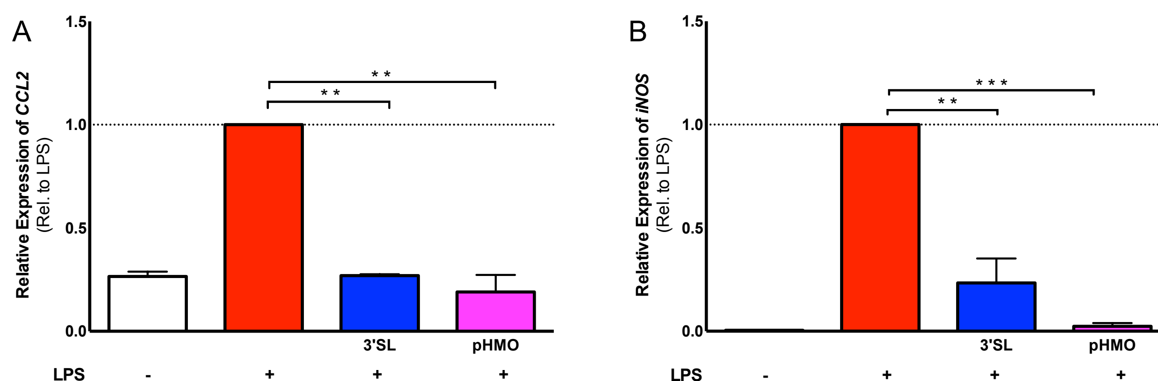


Figure 3.7 Pooled HMO and 3'sialyllactose reduce relative *CCL2* (A) and *iNOS* mRNA expression (B) in LPS-activated murine bone marrow derived macrophages.
BMDM were simultaneously exposed to LPS (10 ng/mL) and either PBS (as intervention control), pHMO (500 µg/mL), or 3'SL (100 µg/mL) for 6 h. Cells cultured in DMEM without LPS and without any HMO served as negative activation control. Data shown are the fold induction of gene expression relative to LPS (10 ng/mL) without HMO treatment (normalized to the housekeeping gene TBP). One way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (** $p<0.01$; *** $p<0.001$); Bars represent mean ± standard deviation; n=2

3.1.7 3'sialyllactose reduces secretion of pro-inflammatory cytokines and additional inflammatory markers in bone marrow derived macrophages

An array-based multiplex Enzyme-Linked ImmunoSorbent Assay (ELISA) system was used to assess and measure the inhibition of various inflammatory markers in the culture medium of LPS-activated BMDM. The pro-inflammatory panel measures ten cytokines that are important in inflammation response and immune system regulation. It detects secreted biomarkers that are implicated in numerous other biological processes, as well as many disorders, including rheumatoid arthritis. Protein concentrations of *IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, CXCL1, IL-10, IL-12p70, and TNF-α,* were quantitatively determined in the cell culture supernatants of BMDM treated for 24 hours with 3'SL or 2'FL (as a negative control), in the presence of LPS (Figure 3.8).

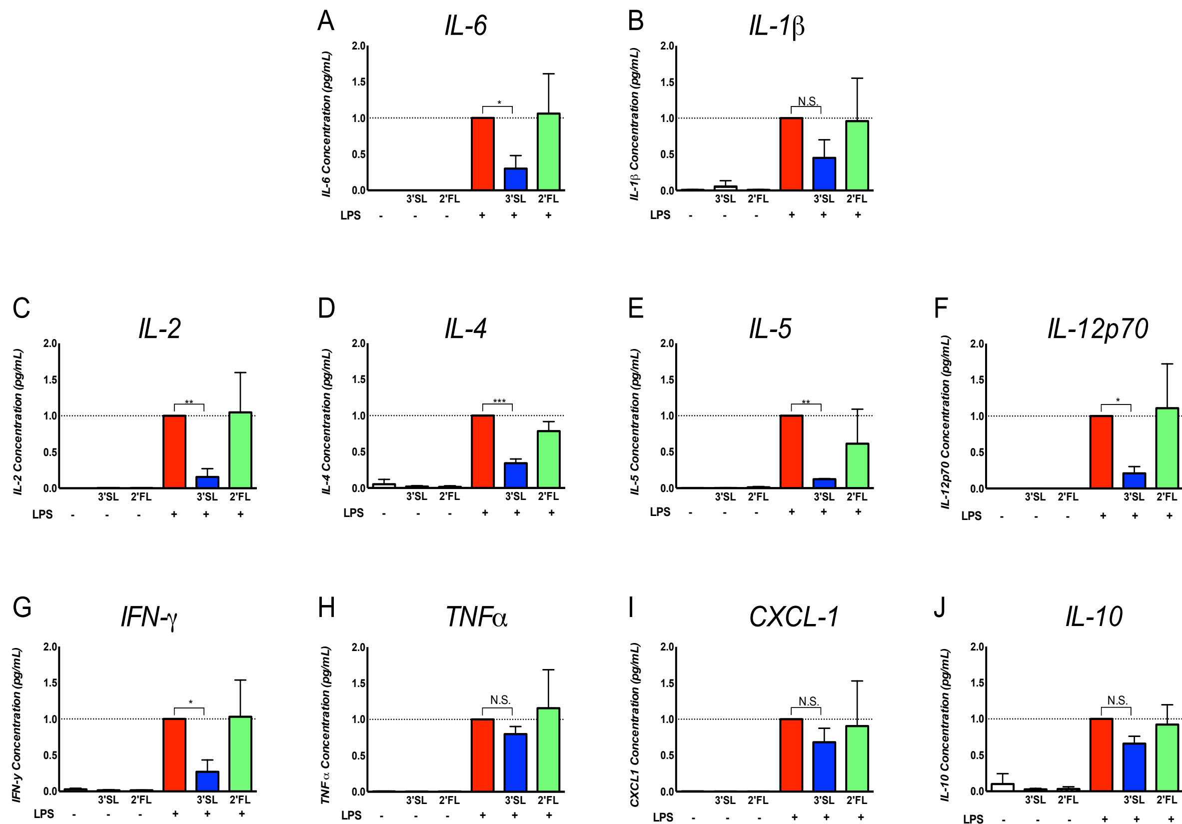


Figure 3.8 3'sialyllactose reduces certain inflammatory cytokine secretions in LPS-activated murine bone marrow derived macrophages.
BMDM were simultaneously exposed to LPS (10 ng/mL) and either PBS (as intervention control), 3'SL or 2'FL (each at 100 µg/mL) for 6 h. Cells cultured in DMEM without LPS and without any 3'SL served as negative activation control. Cytokine concentrations for *IL-6* (A), *IL-1β* (B), *IL-2* (C), *IL-4* (D), *IL-5* (E), *IL-12p70* (F), *IFN-γ* (G), *TNF-α* (H), *CXCL-1* (I), *IL-10* (J) and in the conditioned medium were measured by multiplex ELISA. One way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (* $p<0.05$; ** $p<0.01$; *** $p<0.001$); N.S. = not significant; Bars represent mean ± standard deviation; n=4

As expected, 2'FL (green) did not significantly reduce the secretion of any of the inflammatory biomarkers tested. In addition, 3'SL (blue) did not reduce the concentrations of *IL-10, TNF-α, CXCL1,* or *IL-1β* secreted in the cell supernatants. However, a significant inhibitory effect on secretions of *IFN-γ* (p=0.016), *IL-2* (p=0.009), *IL-4* (p=0.001), *IL-5* (p=0.009), *IL-6* (p=0.02) and *12p70* (p=0.02), was observed when BMDM were treated with 3'SL.

3.2 Identification of responsible mechanism for 3'SL attenuated LPS-induced inflammation in bone marrow derived macrophages

The previously carried out experiments suggest that 3'SL reduces the LPS-induced inflammatory response in macrophages by attenuating the production of various cytokines, such as *IL-6* and *IL-1β*. Potential pathways and receptors capable of interacting with 3'SL were explored to further address the second objective of the study and better understand the mechanisms involved in the anti-inflammatory actions of 3'SL (Figure 3.9).

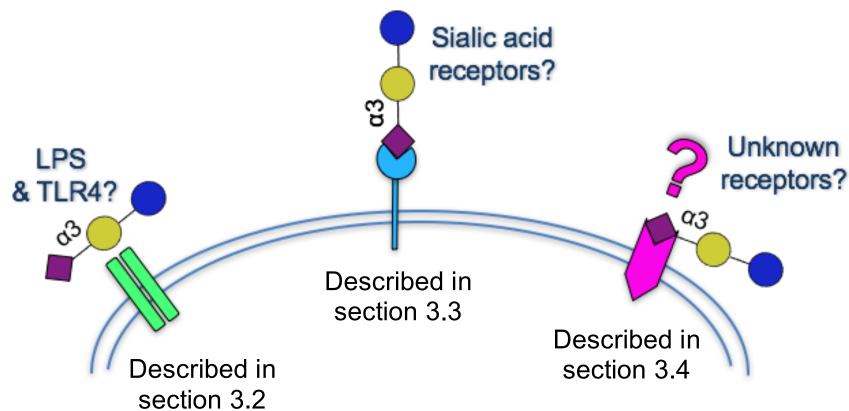


Figure 3.9 Strategies to identify a receptor involved in the mechanisms of 3'SL attenuating pro-inflammatory activation of macrophages.
Interactions of 3'SL with LPS at the TLR4 receptor (described in section 3.2), with sialic acid-binding receptors (described in section 3.3) and with upstream pathway analysis of differentially expressed genes (described in section 3.4) were investigated.

3.2.1 3'sialyllactose attenuates lipid A induced inflammatory response in bone marrow derived macrophages

The interactions of 3'SL with LPS were first investigated in macrophages. LPS is made up of a polysaccharide core and an outer O-Antigen region, with a lipophilic portion, known as lipid A that serves as a membrane anchor (Figure 3.10). The core and O-antigen regions of LPS are composed of glycostructures, mainly of D-glucose and D-galactose amongst others (Miller et al., 2005). The carbohydrate-carbohydrate interactions with 3'SL could potentially interfere with the binding of LPS to surface receptors on macrophages and therefore be responsible for the previously observed anti-inflammatory effects. To rule out the possibility that the reduction in relative expression of pro-inflammatory cytokines *IL-6* and *IL-1β* is a

result of 3'SL interacting with the carbohydrate portion of LPS, BMDM were activated with only the lipid A portion of LPS.

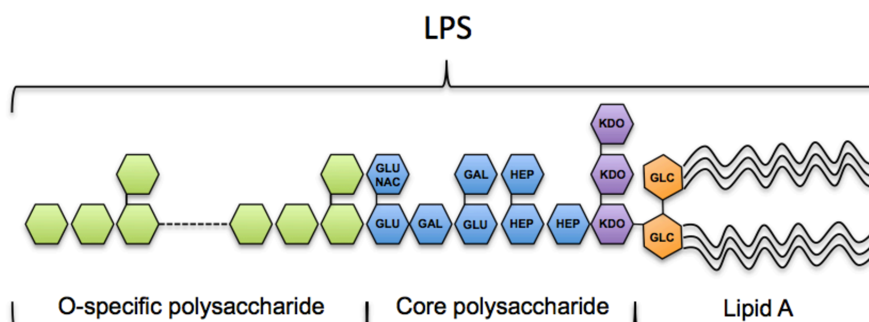


Figure 3.10 A schematic representation of distinct regions of the LPS molecule structure.
The hydrophilic outer O-antigen region consists of repeating tetrasaccharides. The middle core polysaccharide region possesses an overall electronegative charge. The inner lipid A region anchors the molecule into the hydrophobic domain of the bilayer. GLC, glucosamine; KDO, keto-d-octulosonic acid; HEP, heptose; GLU, glucose; GAL, galactose; and GLUNAC, N-acetyl glucosamine.

BMDM were co-stimulated with lipid A and 3'SL for 6 hours. Results indicate that lipid A stimulation (red) increased *IL-6* (Figure 3.11 A) and *IL-1β* (Figure 3.11 B) relative gene expression compared to untreated controls, similarly to previous experiments with LPS stimulation. When cells were simultaneously exposed to lipid A and 3'SL (blue), expression of *IL-6* and *IL-1β* was significantly decreased. The reduction by 3'SL treatment practically attenuated *IL-6* ($p<0.001$), whereas *IL-1β* expression was only reduced to approximately 50% ($p<0.05$), relative to cells stimulated with only lipid A. The observed anti-inflammatory effects of 3'SL on lipid A-activated macrophages suggest that the mechanism of action is independent of carbohydrate interactions.

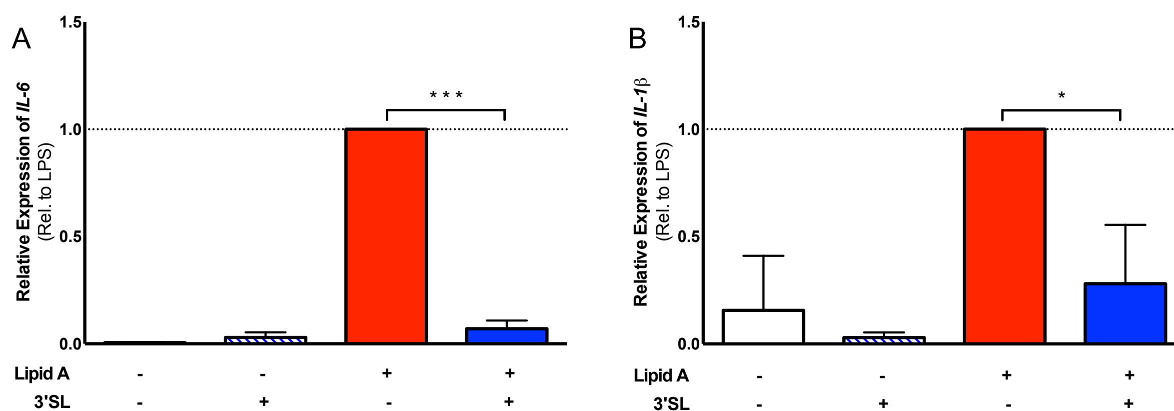


Figure 3.11 3'sialyllactose reduces relative *IL-6* (A) and *IL-1*β mRNA expression (B) in lipid A activated murine macrophage primary cells.
BMDM were simultaneously exposed to lipid A (10 ng/mL) and either PBS (as intervention control), or 3'SL (100 µg/mL) for 6 h. Cells cultured in DMEM without lipid A and without any 3'SL served as negative activation control. Data shown are the fold induction of gene expression relative to lipid A (10 ng/mL) without 3'SL treatment (normalized to the housekeeping gene TBP). One way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (* $p<0.05$; *** $p<0.001$); Bars represent mean ± standard deviation; n=2

3.2.2 3'sialyllactose does not attenuate *IFN-β* induced inflammatory response in bone marrow derived macrophages

Since the likelihood of interactions between 3'SL and the structural component of LPS was excluded, the involvement of the TLR4 pathway was therefore investigated. Upon LPS-activation in macrophages, the TLR4/MD-2 complex can be internalized into endosomes and trigger a chain of signaling cascades (previously described in 1.2.3.1). This helps promote the expression of type I interferons (*IFN)* such as *IFN-β* which are critical cytokines for innate immune responses (reviewed in Mosser & Edwards, 2008) and are implicated in the pathogenesis of chronic inflammatory diseases, such as rheumatoid arthritis (McInnes & Schett, 2007).

To determine if the anti-inflammatory effects of 3'SL are sustained in an *IFN-β* - dependent pathway, BMDM were incubated with *IFN-β* as a stimulus for 6 hours in the presence or absence of 3'SL, and relative gene expression of the pro-inflammatory cytokines *IL-6* and *IL-1β* were determined. Results indicate that exogenous stimulation of macrophages by *IFN-β* (red bar) induced relative gene expression of *IL-6* (Figure 3.12 A), but not *IL-1β* (Figure 3.12 B), compared to the untreated control sample (white bar). Consequently, co-stimulation with *IFN-β* and 3'SL (blue bar) did not attenuate the gene expression of both pro-inflammatory cytokines.

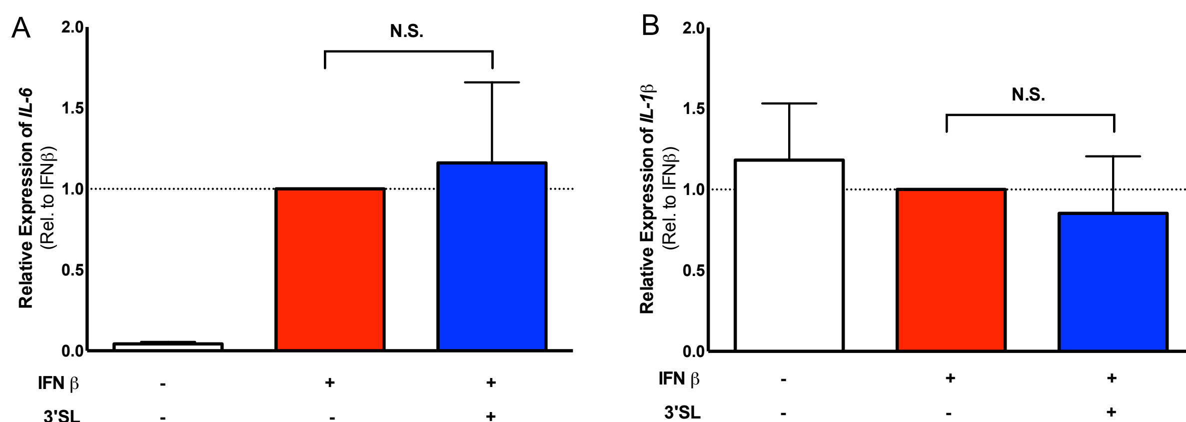


Figure 3.12 3'sialyllactose reduces relative *IL-6* (A) and *IL-1β* mRNA expression (B) in *interferon-β* activated murine macrophage primary cells.
BMDM were simultaneously exposed to *IFN-β* (10 ng/mL) and either PBS (as intervention control), or 3'SL (100 µg/mL) for 6 h. Cells cultured in DMEM without *IFN-β* and without any 3'SL served as negative activation control. Data shown are the fold induction of gene expression relative to *IFN-β* (10 ng/mL) without 3'SL treatment (normalized to the housekeeping gene TBP). One way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments; N.S = not significant; Bars represent mean ± standard deviation; n=3

3.3 Identification of receptors binding 3'sialyllactose responsible for attenuating LPS-induced inflammation, in bone marrow derived macrophages

The prior described experiments did not reveal or confirm that a specific pathway was being employed by 3'SL via LPS/TLR4 signaling. To gain further insight into the potential mechanism involved in the reduction of pro-inflammatory cytokines, one objective was to investigate known receptors on macrophages that could mediate the anti-inflammatory actions of 3'SL.

3.3.1 3'sialyllactose attenuates gene expression of inflammatory cytokines in bone marrow derived macrophages deficient in sialic acid specific-receptors

As previously described (1.2.2.2), lectins known as siglecs are present at the cell surface of macrophages and specifically bind sialic acid moieties to drive anti-inflammatory actions. Since 3'SL features sialic acid at the reducing end of the carbohydrate, experiments were conducted in macrophages lacking Siglec-E or Sialoadhesin receptors. Siglec-E, the murine functional orthologue to the human Siglec-9, was chosen because it preferentially interacts with sialic acid linked in α2-3 position to D-galactose, such as 3'SL (Macauley et al., 2014).

Sialoadhesin shares the substrate-specificity of Siglec-E and has been shown to also bind 3'SL (May et al., 1998), but lacks the intracellular effector component. Sialoadhesin, therefore, served as a negative control (Macauley et al., 2014). BMDM were isolated from mice deficient in Siglec-E and Sialoadhesin-knockout mice (as a negative control), and were activated with LPS and simultaneously treated with 3'SL for 6 hours. The relative mRNA expression of *IL*-6 and *IL-1β* was assessed.

Observations were similar to previous experiments with wild type (WT) BMDM. A significant increase in relative gene expression of *IL-6* was observed in response to LPS stimulation (red bars) in Siglec-E and Sialoadhesin- deficient BMDM (Figure 3.13), when compared to controls (PBS). Both cell types also revealed a significant increase in expression of *IL-6* (Figure 3.13 A and C) and *IL-1β* (Figure 3.13 B and D), indicating an expected inflammatory response to LPS (red bars). In addition, co-stimulation with LPS and 3'SL (blue bars) in BMDM showed a significant decrease in expression of *IL-6* ($p=0.002$) and *IL-1β* ($p=0.015$) compared to LPS-stimulated cells. Results were similar in Sialoadhesin-deficient BMDM and 3'SL significantly reduced expression of *IL-6* ($p<0.001$) and *IL-1β* ($p<0.001$). These findings suggest that Siglec-E is not the receptor responsible for the attenuation of pro-inflammatory-cytokines following the administration of 3'SL.

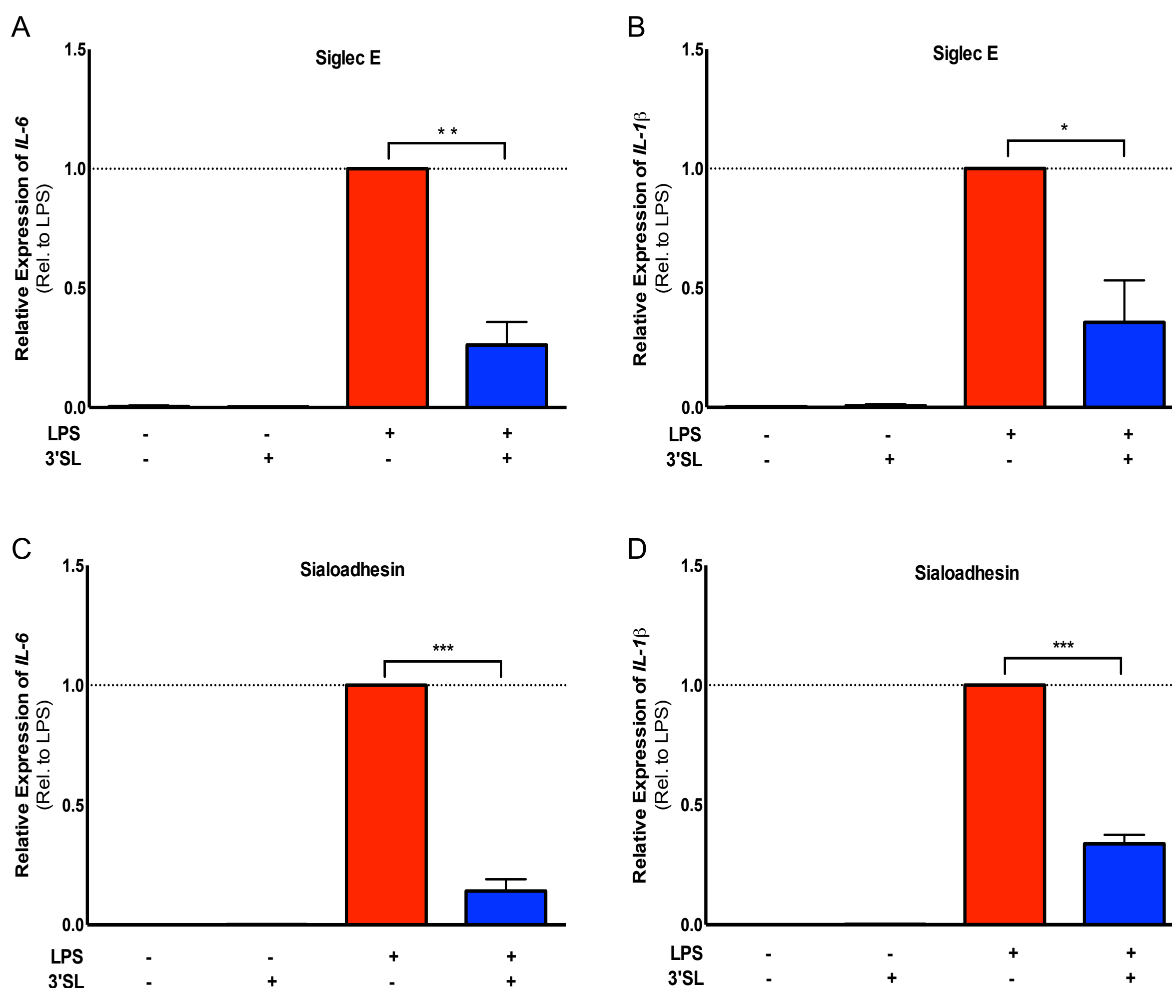


Figure 3.13 3'sialyllactose reduces relative *IL-6* (A, C) and *IL-1β* (B, D) mRNA expression in LPS-activated murine macrophage primary cells, isolated from sialoadhesin (A, B) and siglec-E (C, D) deficient mice.
BMDM were simultaneously exposed to LPS (10 ng/mL) and either PBS (as intervention control) or 3'SL (100 µg/mL) for 6 h. Cells cultured in DMEM without LPS and without 3'SL served as negative activation control. Data shown are the fold induction of gene expression relative to LPS (10 ng/mL) without 3'SL treatment (normalized to the housekeeping gene TBP). One way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (* $p<0.05$; ** $p<0.01$; *** $p<0.001$); Bars represent mean ± standard deviation; n=2

3.3.2 3'sialyllactose attenuates gene expression of inflammatory cytokines in bone marrow derived macrophages deficient in BAI-1 receptor

The engulfment of apoptotic cells and associated pro-inflammatory response by macrophages is mediated by the BAI-1 receptor interacting with gram-negative bacteria through recognition of core oligosaccharide component of LPS at their surface (Soumita Das et al., 2011). Macrophages incubated with apoptotic cells secrete fewer pro-inflammatory and more anti-inflammatory cytokines (Voll et al., 1997). However, de-sialylation of the glycocalyx

of apoptotic cells results in enhanced engulfment and a surge in *TNF-α* and *IL-6* cytokine secretions by macrophages. Sialic acids on the glycocalyx are also thought to be involved in mediating engulfment and cytokine secretions (Meesmann et al., 2010). BMDM isolated from mice in the BAI-1 gene were used for experiments. BAI-1 may also be able to recognize 3'SL which may interfere with the effective binding of LPS to BAI-1. The objective was to investigate whether BAI-1 facilitated the previously observed attenuation of inflammation by 3'SL.

When stimulated with only LPS (red bars), BAI-1-deficient BMDM demonstrated a clear increase in the expression of *IL-6* (Figure 3.14 A) and *IL-1β* (Figure 3.14 B) compared to untreated controls. When LPS was co-administered with 3'SL to BAI-1 deficient cells for 6 hours, a significant decreased in the relative expression in both *IL-6* and *IL-1β* was observed (blue bars), compared to LPS alone (red bars). Altogether, these results indicate that suppressive effects of 3'SL were not connected to an inflammatory response mediated by the BAI-1 receptor.

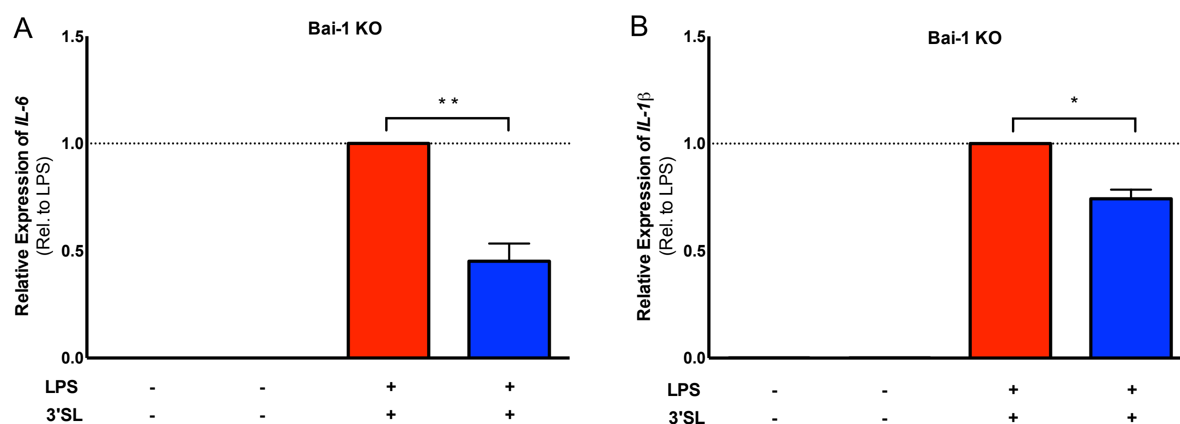


Figure 3.14 3'sialyllactose reduces relative *IL-6* (A), and *IL-1*β mRNA expression (B) in LPS-activated murine macrophage primary cells, isolated from Bai-1-deficient mice.
BMDM cells were simultaneously exposed to LPS (10 ng/mL) and either PBS (as intervention control), or 3'SL (100 µg/mL) for 6 hrs. Cells cultured in DMEM without LPS and without any 3'SL served as negative activation control. Data shown are the fold induction of gene expression relative to LPS (10 ng/mL) without 3'SL treatment (normalized to the housekeeping gene TBP). One way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (* $p<0.05$; ** $p<0.01$); Bars represent mean ± standard deviation; n=2

3.4 Transcriptomic analysis of genes regulated by exposure of 3’sialyllactose in bone marrow derived macrophages

The previously described results suggest that the effects of 3’SL on inflammation were not mediated through direct engagement or competition with the polysaccharide unit of LPS and the TLR4 complex. Additionally, the absence of known sialic acid binding receptors expressed on macrophages (and thus potential ligands for 3’SL) such as Siglec-E, or Sialoadhesin, did not hinder the anti-inflammatory effects mediated by 3’SL. To better understand how 3’SL attenuates LPS-induced inflammation in macrophages, RNA seq analysis was used to compare the transcriptome of BMDM treated with or without LPS or 3’SL and its combinations. BMDM from 3 different mice were used as replicates and were incubated for 6 hours with treatments.

3.4.1 Genes are differentially regulated by 3’sialyllactose stimulation of LPS-activated bone marrow derived macrophages to prevent the activation of inflammatory pathways

A comprehensive view of gene expression changes was elucidated when comparing the transcriptomes of quiescent BMDM (PBS) to BMDM incubated with either 3’SL, LPS, or LPS and 3’SL together. Genes were ranked by fold change (calculated from the logFC), and those with a fold change below 1.5 were excluded from further analysis. The heat map in (Figure 3.15) represents genes (above 2.0-fold) that are significantly up- (in red) and down-regulated (in blue) after LPS stimulation, 3'SL and LPS stimulation, or 3'SL alone, compared to quiescent BMDM. The addition of 3'SL to LPS-stimulated BMDM leads to a similar pattern of differentially regulated genes with slight difference observed (in white), where some genes were not as strongly up- and down-regulated when compared to those same genes in LPS only treated cells. In contrast, very few genes were affected by the treatment of just 3'SL. In fact, these results suggest that a select subset of genes typically up-regulated by LPS or LPS with 3'SL were down-regulated when BMDM were exposed to 3'SL alone.

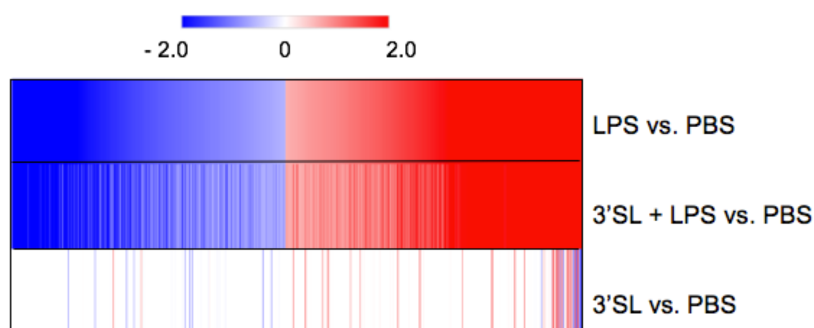


Figure 3.15 Transcriptomic analysis and comparisons of differentially regulated genes by various treatments relative to quiescent (PBS) BMDM. The heat map represents RNA-seq expression of genes differentially up-regulated (red) or down-regulated (blue) in BMDM at 6 h after LPS stimulation, 3'SL and LPS, or 3'SL alone (P < 0.05 and fold change >1.5).

Das et al., (2015) reported that in BMDM over a thousand genes are differentially expressed (>1.5 fold) in response to a 4-hour exposure to LPS. In line with these results, RNA-seq analysis revealed that nearly 6000 genes (2977 up and 2847 down-regulated) were affected when the BMDM were incubated with LPS for 6 hours when compared to BMDM stimulated with PBS. In comparison, only 48 and 85 genes were up- and down-regulated, respectively when cells were treated with 3'SL during LPS activation, compared to the genes affected by LPS without 3'SL treatment (Supplemental 3 and Supplemental 4). Data sets of genes that were significantly down- and up-regulated by 3'SL in LPS-treated cells were compared to up- and down-regulated genes by 3'SL in basal media (Supplemental 1 and Supplemental 2). The results indicate that 307 genes were up- and 156 genes were down regulated by 3'SL alone. Out of the 48 up- and 85 down-regulated genes by 3'SL in the presence of LPS, only 12 and 2 genes (GYPC and BCL2ALB) were found to be similarly affected in both data sets, respectively (Figure 3.16). A total of 133 genes were differentially regulated in BMDM exposed to the 3'SL and LPS co-treatment, compared to 5823 genes from the LPS treatment. These results suggest that 3'SL has a selective impact on total gene expression changes related to LPS stimulation. These results support the notion that 3'SL does not exert its anti-inflammatory effects by directly inhibiting the LPS-initiated TLR4 activation.

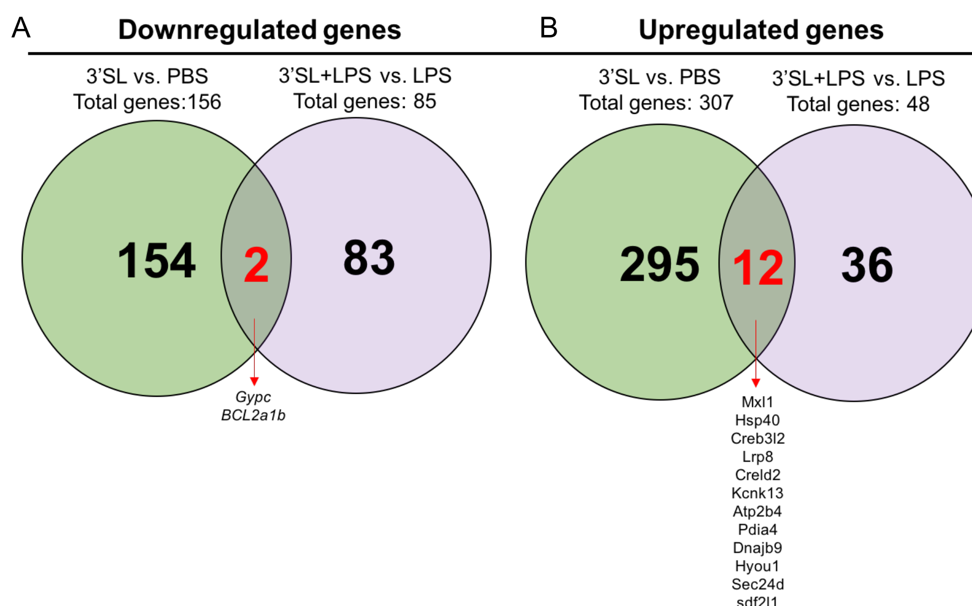


Figure 3.16 Venn-diagrams represent the number of differentially expressed genes detected gene list in RNA-seq data analysis.
Green figures indicate differentially expressed genes down-regulated by 3'SL compared to non-treated control (PBS). Purple figures indicate differentially expressed genes down-regulated by 3'SL in the presence of LPS compared to non-treated LPS-stimulated cells (A). Green figures indicate differentially expressed genes up-regulated by 3'SL compared to non-treated control (PBS). Purple figures indicate differentially expressed genes up-regulated by 3'SL in the presence of LPS compared to non-treated LPS-stimulated cells (B). Diagrams show the shared number of differentially expressed genes (in red) that are down or up-regulated (A and B). Adjusted p-value < 0.10 is used as cut-off for comparisons among different treatments.

In the presence of LPS 3'SL significantly down-regulates the expression of pro-inflammatory genes, where *Cxcl3* and *IL-6* were most significantly affected, with greater than 4-fold decrease in expression when compared to non-treated LPS-stimulated BMDM (Supplemental 3). Of the genes that were significantly up-regulated by 3'SL in the presence of LPS the highest increase (by 2.97 fold) was observed for the expression in low-density lipoprotein receptor-related protein 8 (LRP8) (Supplemental 4). This gene encodes for the apolipoprotein E (apoE) receptor, known for promoting cholesterol efflux and lipoprotein clearance to exert anti-atherogenic effects (Nakashima et al., 1994) and may play a significant role in the observed anti-inflammatory effects of 3'SL.

In order to provide further biological insight into the observed expression changes, upstream regulatory molecules and associated mechanisms were identified using the Ingenuity Pathway Analysis software (IPA) (Qiagen Bioinformatics). Results indicate that 3'SL activated

the prostaglandin E receptor 4 (PTGER4) most significantly, with the highest Z-score (3.4) and p-value overlap <0.001 in LPS-stimulated BMDM (Supplemental 5). Prostaglandin E2 (PGE_2) exerts anti-inflammatory effects by binding to PTGER4 to modulates innate and adaptive immunity, tissue remodeling and repair in macrophages. The activation of PTGER4 is shown to suppress macrophage cytokine and chemokine secretions (Tang et al., 2012). As expected, LPS and pro-inflammatory mediators such as *IL-1β* and *TNF* were significantly inhibited (z-score ranging from 4.18 to 3.7) by 3'SL exposure to BMDM stimulated with LPS (Supplemental 6). In addition amyloid precursor protein (APP), which functions as a modulator of the peripheral immune system and lipid metabolism (Puig et al., 2017) was also significantly inhibited (z-score = 3.89) compared to non-treated, LPS-activated BMDM.

To further understand the association between the observed gene expression changes and the upstream regulatory molecules, the canonical signal transduction or metabolic pathways involved in macrophages treated with LPS and LPS with 3'SL were determined using the IPA software. The involvement of 47 pathways were predicted to be significant ($p<0.05$) and the 20 most significant were presented (Figure 3.17). Results suggest that exposure of 3'SL to LPS-stimulated BMDM is associated with inhibiting the pathway for Acute Phase Response which serves as the core of the innate immune response, thereby initiating the inflammatory processes (Cray et al., 2009). 3'SL also inhibited the pathway for high mobility group box 1 (HMGB1) signaling. LPS induces HMGB1, which modulates the inflammatory cascade in activated macrophages while suppressing an anti-inflammatory response (El Gazzar, 2007) and facilitates macrophage polarization towards M1-like phenotype via the TLR4-PI3Kγ-Erk1/2 pathway (Su et al., 2016). Results also indicate that 3'SL engages the pathway for LXR/RXR Activation. The liver X receptor (LXR) is activated by oxysterol ligands and forms a heterodimer with the retinoid X receptor (RXR) and together are involved in the regulation of cholesterol, lipid metabolism, and inflammation (reviewed in Tall & Yvan-Charvet, 2015).

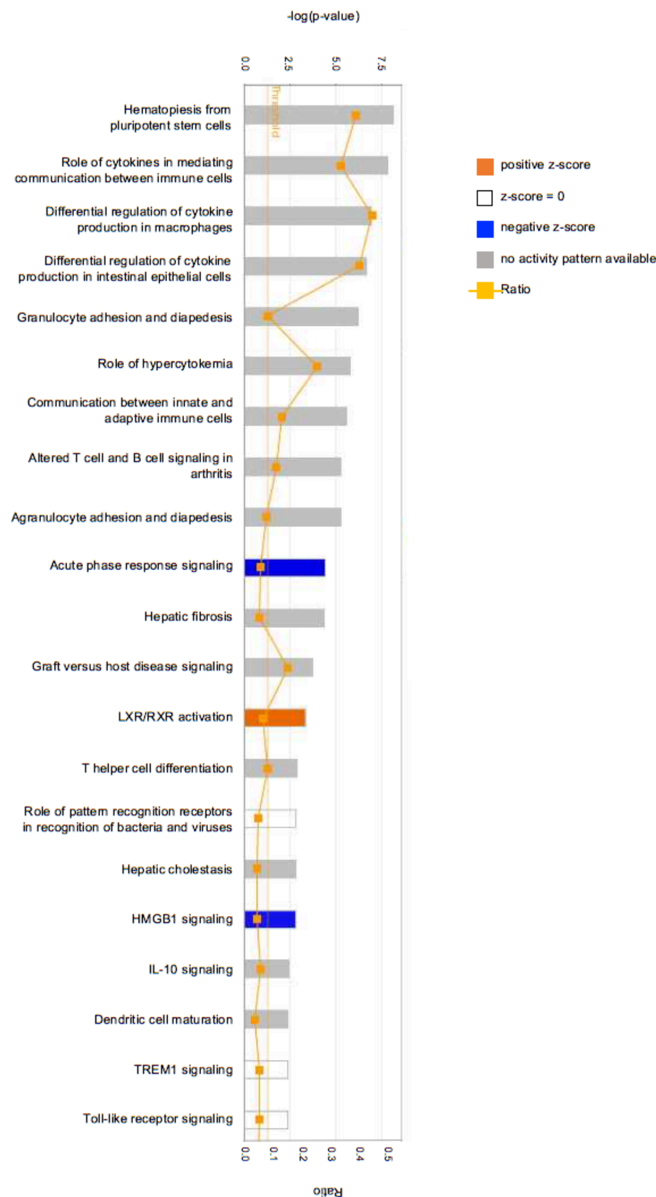
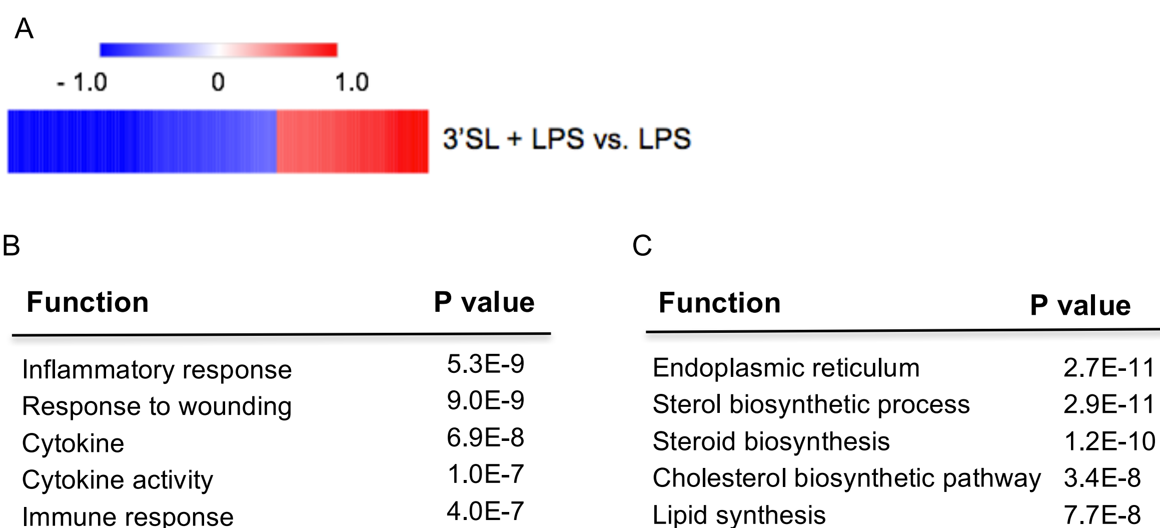


Figure 3.17 The canonical pathways analysis shows the significant pathways between LPS-stimulated macrophages with and without 3'SL.
Pathways identified are represented on the x-axis and listed according to their p value (-Log). The left y-axis corresponds to the –log of the p-value (Fisher's exact test) and the orange threshold line corresponds to a –log (p-value)>1.3 or a p-value of 0.05. The right y-axis represents the ratio of list genes found in each pathway that meet cutoff criteria (fold change >2 and p-value <0.05), over the total number of genes in that pathway (Ratio, orange squares). The most statistically significant canonical pathways identified are shown according to their Z-score (negative: blue bars; positive: orange bars).

The effects of 3'SL in the presence of LPS were evaluated and compared to BMDM exposed to only LPS. The heat map in (Figure 3.18 A) represent genes (above 1.0-fold) that are significantly up- (in red) and down-regulated (in blue) after co-incubation with 3'SL and LPS. Results suggest that the addition of 3'SL leads to an increase in down-regulated genes, compared to up-regulated genes (Figure 3.18 A). To further functionally classify the

differentially regulated genes, the web-accessible program known as the Database for Annotation, Visualization and Integrated Discovery (DAVID), was used an analytic tool to confirm the functional annotation of the gene data set by the IPA software. Significantly up- and down-regulated genes by 3’SL in LPS-stimulated BMDM were analyzed separately. The integrated biological knowledgebase of DAVID contained data sets from thousands of public studies that inform biological interpretation and revealed that the large number of genes that are differentially down-regulated by 3’SL are involved in inflammatory and immune processes (Figure 3.18 B). Interestingly, the differentially up-regulated genes are involved in lipid and cholesterol synthesis (Figure 3.18 C), which confirms the involvement of the LXR and RXR pathway previously determined by the IPA software analysis. The up-regulation in expression of genes that mediate cholesterol metabolism could contribute to the downregulation of genes associated with inflammation for the resolution phase of the TLR4 inflammatory response (Oishi et al., 2017) and may provide insight into how 3’SL attenuates LPS stimulation in BMDM.



B

Function	P value
Inflammatory response	5.3E-9
Response to wounding	9.0E-9
Cytokine	6.9E-8
Cytokine activity	1.0E-7
Immune response	4.0E-7

C

Function	P value
Endoplasmic reticulum	2.7E-11
Sterol biosynthetic process	2.9E-11
Steroid biosynthesis	1.2E-10
Cholesterol biosynthetic pathway	3.4E-8
Lipid synthesis	7.7E-8

Figure 3.18 Transcriptomic analysis (A) and functional annotation of genes down-regulated (B) or up-regulated (C) by 3’SL in LPS-stimulated BMDM.
The heat map represents differential expression genes in BMDM stimulated with 3’SL and LPS for 6 h, compared to cells stimulated with only LPS (A). 3’SL decreases expression of inflammatory genes (B) and increases expression of lipid synthesis genes (C) in LPS-activated murine bone marrow derived macrophages p-values (<0.01) of selected differentially regulated gene sets (>1.5 fold) in 3’SL treated BMDM, identified by DAVID.

3.4.2 The transcription factor binding sites in the promoter regions of genes differentially expressed by 3'sialyllactose stimulation of LPS-activated bone marrow derived macrophages

To provide an additional approach to identifying the dominant ligand-receptor pathway activated and/or inhibited by 3'SL, the promoter regions of the differentially expressed genes were scanned to identify transcription factor binding sites using the web-based Motif discovery tool HOMER (Hypergeometric Optimization of Motif EnRichment) (Heinz et al., 2010). The software generated a sorted list of non-redundant motifs ranked by their enrichment p-values (Table 3.2 and Table 3.3). The promoter analysis displayed the most relevant transcription factor binding sites regulating the ALX/FPR2 gene. Results were cross-referenced to literature and UCSC Genome Browser, in addition to work by Waechter et al. (2012), which identified SP1 as a putative transcription factor binding site involved in regulating transcription of ALX/FPR2 in macrophages. ALX/FPR2 is a G protein-coupled receptor belonging to the formyl peptide receptor (FPR) family for ligands with various biological functions, such as lipoxin A4 and annexin A1 (AnxA1), which are pro-resolution mediators. In addition, the pro-inflammatory protein serum amyloid A (SAA), which was shown to be significantly down-regulated by 3'SL in the RNA-seq analysis (Supplemental 3) is known to stimulate foam cell formation in macrophages via ALX/FPR2 signaling and LOX1 induction (Bena et al., 2012; Dufton et al., 2010; Ha Young Lee et al., 2013).

Table 3.2 Transcription factor binding sites for genes down-regulated by 3'sialyllactose in LPS-stimulated BMDM

% of Targets	% of Background	Transcription factor binding sites
17.3	0.8	NFkB-p65-Rel
14.6	0.9	PU.1
29.3	7	DCE_S_III
14.7	1.5	Klf4(Zf)
9.3	0.5	Fox:Ebox
10.7	0.8	DCE_S_II
12	1.5	HNF6
26.7	8.3	ETS
21.3	5.5	AC
21.3	5.7	Tbx5
42.7	19.8	Pbx1
8	0.6	MyoD(bhlH)
5.3	1.1	Foxa2_2

Table 3.3 Transcription factor binding sites for genes up-regulated by 3'SL in LPS-stimulated BMDM

% of Targets	% of Background	Transcription factor binding sites
6.8	0.4	NFYB
10.2	1.4	RUNX1
6.8	0.5	Nr2e3
10.2	1.6	NFY(CCAAT)
7.4	0.7	MafA
7.4	0.7	Atf4
35.2	16.9	Sox9
6.8	0.7	Bhlhe40
10.8	2	MYC:MAX
10.2	2	E2F1
8.5	1.5	CCAAT-box
4.6	0.3	SOX10
6.3	0.8	Smad3(MAD)
23.3	10.9	TATA box (TBP)
18.8	8.1	DCE_S_I
5.1	0.8	RFX5

3.5 Identification of candidate target receptors and pathways involved in 3'sialyllactose attenuation of LPS-induced inflammatory response in macrophages

Geneontology and pathway analysis of RNA-seq data revealed that genes affected by 3'SL stimulation were involved in macrophage lipid synthesis and cholesterol homeostasis, which are known to modulate macrophage polarization and promote resolution (Medbury, 2015). Evidence suggests that ALX/FPR2 or EP4 are possible receptors involved in this process to support the anti-inflammatory effects observed in 3'SL treatment of LPS-stimulated BMDM. Antagonist and agonist assays were performed to further elucidate the mechanism and the participation of these candidate receptors.

3.5.1 Expression of ALX/FPR2 receptor in LPS-stimulated murine macrophage cell line and primary cells.

The expression levels of ALX/FPR2 mRNA were determined by qRT-PCR, and the effects of LPS and 3'SL on the receptor's relative expression were revealed. The macrophage cell line Raw 264.7 and primary cells (BMDM) were stimulated with LPS or PBS as a baseline control for 6 hours, in the presence or absence of 3'SL. LPS-activated cells in Raw 264.7 (Figure 3.19 A) and in BMDM (Figure 3.19 B) and significantly increased ($p<0.01$). ALX/FPR2

relative mRNA expression (red bars) compared to non-stimulated cells. The addition of 3'SL to basal medium did not affect ALX/FPR2 relative mRNA expression. However, it was significantly decreased ($p<0.05$) by co-incubation of 3'SL and LPS (bleu bars) in Raw 264.7 (Figure 3.19 A) and in BMDM (Figure 3.19 B), compared to cells just treated with LPS.

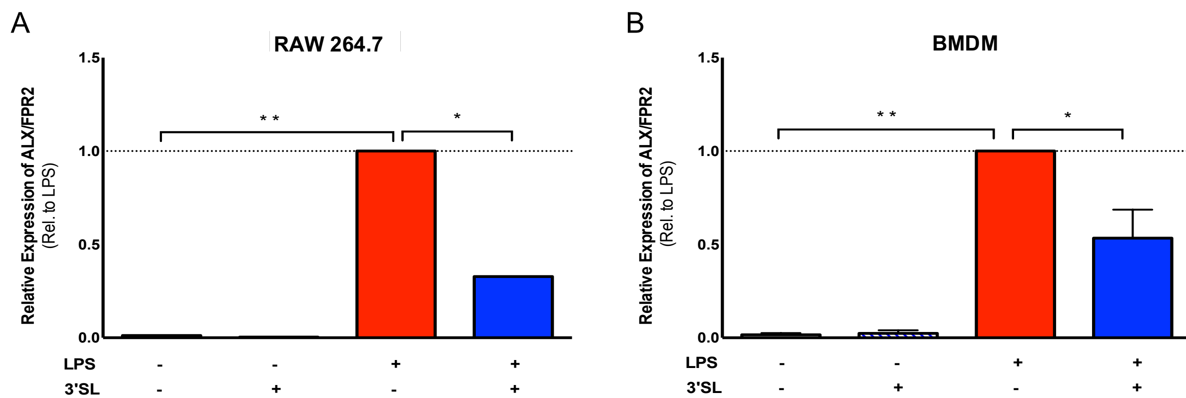


Figure 3.19 3’sialyllactose reduces relative ALX/FPR2 mRNA expression in LPS-activated murine macrophage cell line (A) and in primary cells (B).
Cells were simultaneously exposed to LPS (10 ng/mL) and either PBS (as intervention control), or 3’SL (100 µg/mL) for 6 h. Cells cultured in DMEM without LPS and without any 3’SL served as negative activation control. Data shown are the fold induction of gene expression relative to LPS (10 ng/mL) without 3’SL treatment (normalized to the housekeeping gene TBP). One way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (* $p<0.05$; ** $p<0.01$); Bars represent mean ± standard deviation; n=2

3.5.2 ALX/FPR2 receptor antagonists do not attenuate 3’sialyllactose mediated inhibition of LPS inflammation in murine bone marrow derived macrophage

Studies show that the specific antagonist Trp-Arg-Trp-Trp-Trp-Trp-CONH2 (WRW4) can block ALX/FPR2 and induces a high *IL-6* response in addition to LPS stimulation of macrophages (Kao et al., 2014). Another antagonist known as N-t-BOC-Phe-Leu-Phe-Leu-Phe (BOC2) has been shown to reverse the potent anti-inflammatory effects of Resolvin D1 (RvD1) via the ALX/FPR2 (Li et al., 2014; Odusanwo et al., 2012). Relative mRNA expression of *IL-6* was measured by qRT-PCR, to assess if the anti-inflammatory effects of 3’SL were hindered in the presence of these ALX/FPR2 antagonists. BMDM were pre-incubated with ALX/FPR2 antagonists WRW4 (Figure 3.20 A) or BOC2 (Figure 3.20 B) at concentrations of 10 µM or 50 µM, for 30 min. Cells were then stimulated for an additional 6 hours with LPS, LPS with 3’SL, 3’SL alone, or PBS as a control. The addition of 3’SL to

BMDM incubated with 3'SL, LPS, and WRW4 at 10 µM (white bar, blue outline) or 50 µM (blue shaded bar, blue outline) significantly decreased relative *IL-6* mRNA expression compared to LPS and WRW4 at 10 µM (white bar, red outline) (p<0.05) or LPS and WRW4 at 50 µM (red shaded bar, red outline) (p<0.05). Similar results were observed with ALX/FPR2 antagonist BOC2 where 3'SL inhibited the relative mRNA expression of *IL-6* in cells treated with 10 µM of BOC2 (white bar, blue outline) or 50 µM (blue shaded bar, blue outline) in the presence of LPS (p<0.05).

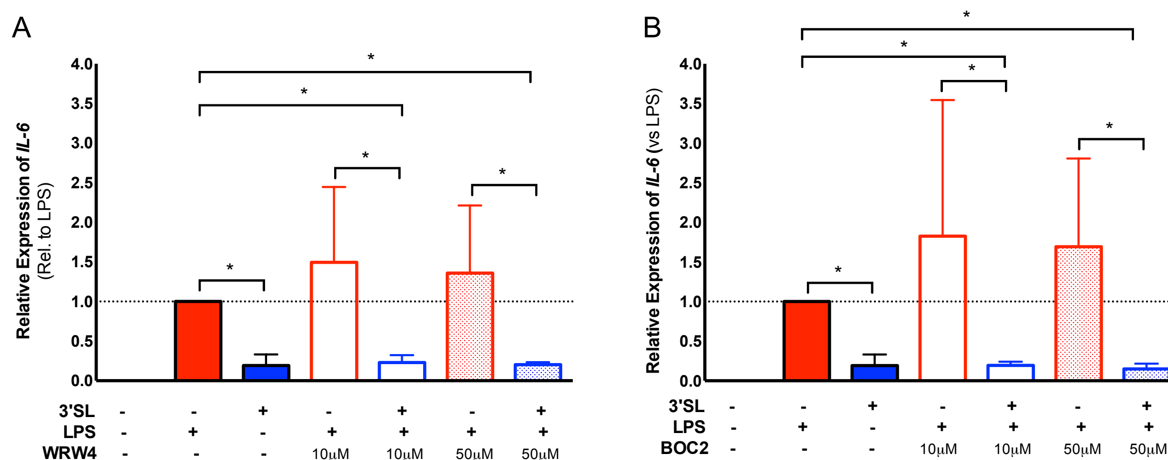


Figure 3.20 3'sialyllactose reduces relative *IL-6* mRNA expression in LPS-activated murine macrophage primary cells, when pre-incubated for 30 min with ALX/FPR2 antagonists WRW4 (A) or BOC2 (B) (each at 10µM or 50µM). Cells were simultaneously exposed to LPS (10 ng/mL) and either PBS (as intervention control), or 3'SL (100 µg/mL) for 6 h. Cells cultured in DMEM without LPS and without any 3'SL served as negative activation control. Data shown are the fold induction of gene expression relative to LPS (10 ng/mL) without 3'SL, WRW4 or BOC2. Treatments were normalized to the housekeeping gene TBP. ANOVA and multiple comparisons tests was used to show the statistical significance between treatments (* p<0.05) Bars represent mean ± standard deviation; n=3

3.5.3 3'sialyllactose mediated inhibition of LPS inflammation is not attenuated by EP4 receptor antagonist however it is enhanced by EP4 receptor agonist

Results suggest that EP4 is involved mainly in the inhibition of cytokine release in macrophages (Ikegami et al., 2001) and that LPS-induced production of inflammatory mediators were attenuated by EP4 agonist (Sakamoto et al., 2004). In addition, results show that the EP4 receptor antagonist blocks the anti-inflammatory activity of both the selective EP4 receptor agonist and PGE_2 itself (Birrell et al., 2015). The impact of specific EP4 receptor antagonists (ONO-AE3-208) (Figure 3.21 A) and agonists (CAY10598) (Figure 3.21

B) were studied with LPS-stimulated BMDM to determine if the anti-inflammatory effects of 3'SL could be prevented. Relative mRNA expression of *IL-6* was measured by qRT-PCR, after pre-incubation of cells for 30 min with EP4 agonist and antagonist at concentrations of 10 nM or 100 nM, in the presence or absence of LPS and 3'SL, for an additional 6 hours and PBS was used as the vehicle. LPS-stimulated BMDM treated with 3'SL revealed a significant decrease in relative *IL-6* expression in the presence of EP4 antagonist at 10 nM (shaded blue bar) ($p<0.05$) and 100 nM (lined blue bar) ($p<0.05$), when compared to LPS (red bar) (Figure 3.21 A). In contrast, although no statistical significance could be determined (n=1), the addition of EP4 agonist (Figure 3.21 B) reduced the relative expression *IL-6* at concentrations of 10 nM (blue shaded bar, blue outline) and 100 nM blue lined bar, blue outline) compared to LPS treated cells (red bar). The addition of 3'SL and antagonist at 100 nM to LPS-stimulated cells (white bar blue outline) was most effective in reducing *IL-6* relative expression, suggesting a possible additive effect acting through the EP4 receptor.

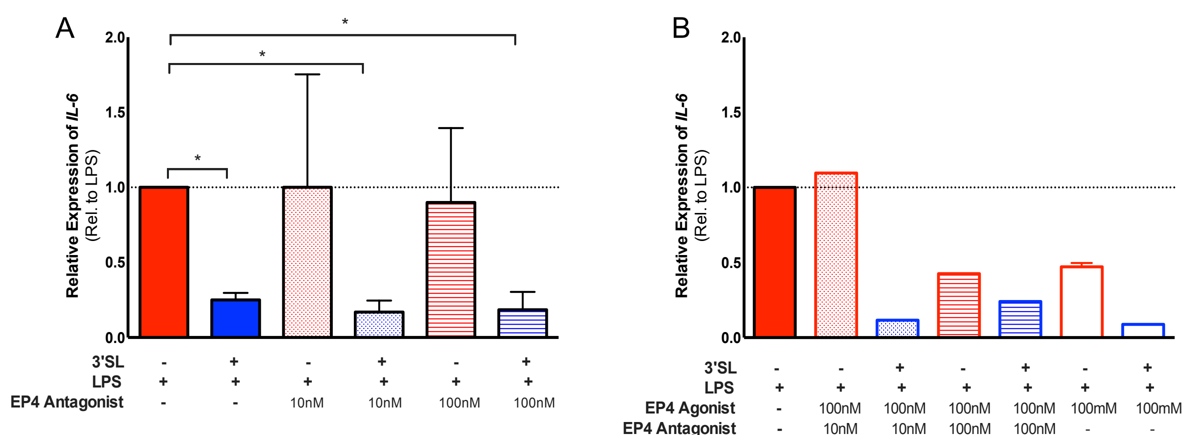


Figure 3.21 3'sialyllactose reduces relative EP4 mRNA expression in LPS-activated murine macrophage primary cells, when pre-incubated for 30 min with EP4 antagonist (10nM or 100nM) (A) or EP4 agonist (100nM) or in combination with EP4 antagonist (10nM or 100nM) and EP4 agonist (100nM) (B).

Cells were simultaneously exposed to LPS (10 ng/mL) and either PBS (as intervention control), or 3'SL (100 µg/mL) for 6 h. Cells cultured in DMEM without LPS and without any 3'SL served as negative activation control. Data shown are the fold induction of gene expression relative to LPS (10 ng/mL) without 3'SL, EP4 antagonist, or EP4 agonist treatments (normalized to the housekeeping gene TBP). One way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (* $p<0.05$); Bars represent mean ± standard deviation; n=1-2

3.5.4 3'sialyllactose mediated inhibition of LPS inflammation is attenuated by EP4 receptor silencing in Raw 264.7 macrophages

EP4 antagonist assays were not effective in blocking the 3'SL mediated inflammation in BMDM. However, there was an observed additive effect in LPS-stimulated BMDM simultaneously exposed to both the EP4 agonist and 3'SL. Commonly used small interfering RNA (siRNA) technology (Hamilton, 1999; Steenport et al., 2009) was employed to silence the gene for the EP4 receptor. The loss of the candidate gene would presumably result in an anticipated loss of the 3'SL anti-inflammatory properties and would confirm the involvement of the EP4 receptor. EP4 expression was silenced in RAW 264.7 macrophages with siRNA and cells were subsequently treated LPS in the absence or presence of 3'SL, for 6 hours. Knockdown efficiency was monitored using qRT-PCR for EP4 mRNA. Levels of EP4 mRNA were markedly decreased (by approximately 50%) in LPS-activated RAW264.7 cells, transfected with EP4 siRNA (siEP4, blue striped bars) when compared to nonspecific siRNA-transfected (siSCR, red striped bars) cells (Figure 3.22 A).

EP4 siRNA transfection resulted in an increased *IL-6* expression by RAW264.7 macrophages (Figure 3.22 B) when stimulated with LPS (red bars), when compared to non-treated cells (white bars). The ability of 3'SL to reduce *IL-6* expression was verified in LPS-stimulated nonspecific siRNA-transfected cells (siSCR, blue bars) and revealed a significant decrease in expression when compared to cells only exposed to LPS nonspecific siRNA-transfected cells (siSCR, red bars). In contrast, the ability of 3'SL to reduce *IL-6* expression in EP4 transfected cells (siEP4 blue bars) was hindered when compared to cells only treated with LPS alone (siEP4, red bars). As predicted, 3'SL was unable to reduce *IL-6* expression by EP4 knockdown cells. From these results, it appears that the EP4 receptor may mediate the observed anti-inflammatory effects of 3'SL.

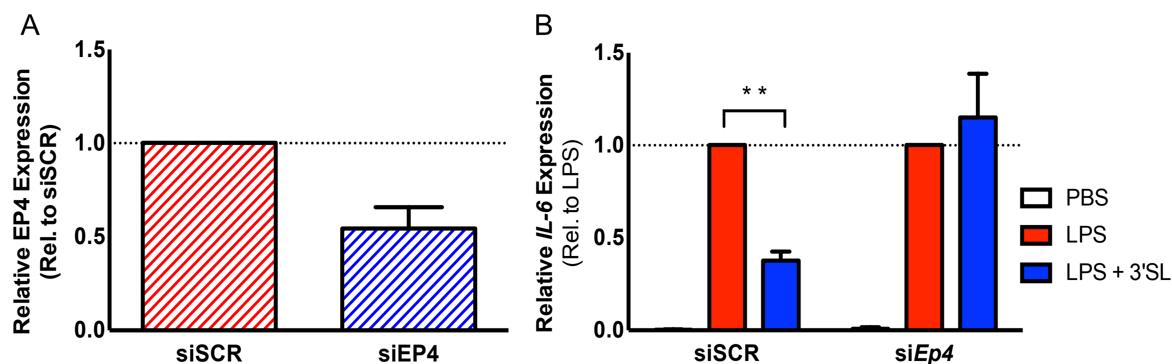


Figure 3.22 EP4 mRNA expression in LPS-activated RAW 264.7 cell line (A) is reduced by siRNA transfection (A) and EP4 silencing inhibits the reduction of *IL-6* expression by 3'sialyllactose (B).
Cells were simultaneously exposed to LPS (10 ng/mL) and either PBS (as intervention control), or 3'SL (100 µg/mL) for 6 h. Cells cultured in DMEM without LPS and without any 3'SL served as negative activation control. Data shown are the fold induction of gene expression relative to LPS (10 ng/mL) without 3'SL treatment (normalized to the housekeeping gene TBP). One way ANOVA tested for multiple comparisons was used to show the statistical significance between treatments (** $p<0.01$); Bars represent mean ± standard deviation; n=2

3.6 Impact of 3'sialyllactose on lipid metabolism and foam cell conversion of murine bone marrow derived macrophage

The RNA-seq data revealed that 3'SL up-regulated the *LRP8* gene, which encodes for the apolipoprotein E receptor-2 (apoER2). Research suggests that it may play a significant role in atherosclerosis development and progression and that macrophages deficient in apoER2, accumulated more lipids and were more susceptible to oxidized LDL (oxLDL)-induced death (Waltmann et al., 2014). In addition, gene ontology analysis using DAVID and IPA highlighted the potential importance of 3'SL in regulating genes involved in cholesterol homeostasis. Previous results suggest that addition of 3'SL significantly attenuates LPS-induced inflammation in macrophages supporting the possibility that it can also attenuate inflammation driven by modified Low Density Lipoproteins (LDL), which similarly to LPS, uses the TLR4 to activate macrophages (Stewart et al., 2010). The complex disease process of atherosclerosis is characterized by the accumulation of lipids and inflammatory cells in arteries (Libby et al., 2013). Increasing levels of circulating LDL are trapped and modified into oxidized LDL (oxLDL), thereby inducing attraction and binding of monocytes (Yutaka Nakashima et al., 2008). In turn, monocytes differentiate into macrophages and internalize oxLDL and aggregate LDL (agLDL) particles and accumulate massive amounts of cholesterol to become lipid droplet-loaded cells known as foam cells. The amplified a chronic

inflammatory response resulting from foam cell formation promotes the continuous flow of M1 macrophage secreting cytokines and chemokines are of primary etiological importance in all stages of atherosclerosis (Kathryn J. Moore & Tabas, 2011). Over time, the system becomes locked in a state of non-resolving chronic inflammation and results in significant clinical consequences, leading to fatality (Lusis, 2000).

Considering the critical role of chronic inflammation and accumulation of M1 macrophages in atherosclerosis, the effects of 3'SL on foam cell formation were assessed *in vitro*. BMDM were stimulated with 50 µg/mL oxLDL or agLDL in the presence of 3'SL for 24 hours (Figure 3.23). As expected, cholesterol esters, which are a measure of foam cell conversion, were not detectable in non-LDL stimulated (PBS) cells, in the presence or absence of 3'SL. In addition, BMDM activated by ox-LDL did not produce detectable concentrations of cholesterol esters in either 3'SL-treated (blue) or PBS-treated cells. However, cells were activated by agLDL resulting in a significant production of cholesterol esters in non-treated cells (white bar). In comparison, the addition of 3'SL (blue bar) significantly ($p<0.05$ value) reduced the accumulation of cholesterol esters in BMDM. These results indeed indicate that 3'SL can attenuate agLDL induced foam cell formation and support a possible role of 3'SL in modulating foam cell conversion.

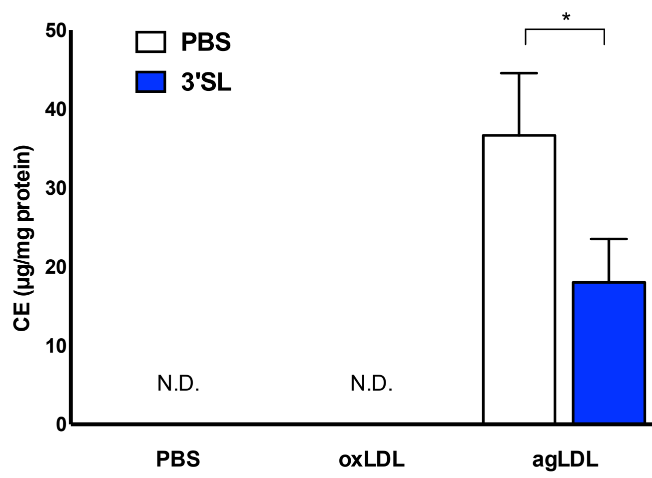


Figure 3.23 3'sialyllactose reduces cholesterol ester accumulation in foam cell induced - bone marrow derived macrophages.
Cholesterol ester levels in BMDM were incubated with 0 or 50 µg/mL of oxLDL or agLDL (n=3) to induce foam cell formation. Cells were simultaneously treated with 100 µg/mL 3'SL (blue bars), or PBS as a control (white bars), for 24 hours. Cells cultured in DMEM without LDL and with or without any 3'SL served as negative activation control. Cholesterol esters were determined by subtracting free cholesterol from total cholesterol. Data shown are total concentrations of cholesterol ester in the conditioned media, quantified by fluorometric detection and normalized to total protein of cell lysate per treatment. T-test was used to show the statistical significance between treatments (*$p<0.05$); Bars represent mean ± standard deviation; n=3; N.D. = Not detectable

3.7 Efficacy of 3'sialyllactose in attenuating LPS driven inflammation, *in vivo*

Results in the *in vitro* models support the notion of 3'SL as a potential anti-inflammatory agent. The effects of 3'SL were observed in various types of macrophages and backed up by RNA-seq, which unraveled a multitude of inflammatory genes down-regulated by 3'SL. An *in vivo* approach was taken to address the fifth aim of the study, to investigate the inhibitory effects of 3'SL in an inflammatory disease model.

3.7.1 Variation of 3'sialyllactose pharmacokinetics in murine *in vivo* models

In order to uncover the therapeutic effects of 3'SL *in vivo*, the best route of administration was determined by analyzing the pharmacokinetics of 3'SL in mice. Blood was collected from mice given a single dose of 3'SL via various means such as intraperitoneal injection (i.p.), intravenous injection (i.v.) or by oral gavage for intragastric administration (i.g.).

Blood samples were collected from the tail vein serially before (0) and at 5, 10, 15, 30, 60 and 180 min after IP (250 µg of 3'SL) or IV administration (100 µg 3'SL). Within 5 min of IP

injection (Figure 3.24 A) 3'SL appeared in blood, peaked at 10 min and returned to the baseline within 60 to 180 min. When injected intravenously (Figure 3.24 B), 3'SL peaked at 5 min and returned to baseline between 30 to 60 minutes.

For oral 3'SL treatment, mice were divided into three groups, given a bolus dose by oral gavage of either 30, 60 or 90mg of 3'SL (Figure 3.24 C) and blood samples were collected from the tail vein serially before (0) and at 10 15 30 45 60 and 180 min. Concentrations of 3'SL in blood peaked at 45 min in mice receiving 30mg of 3'SL (green line) and 30 minutes in mice receive 60mg (blue line). In both treatments 3'SL returned to baseline within 60 min. In contrast, 3'SL peaked at 15 min in mice receiving 90 mg of 3'SL (pink line) and remained elevated at 60 min and returned to baseline by 180 min.

To determine if a fixed concentration of 3'SL could be maintained systemically, the effects of continuous administration were observed by mixing 3'SL with drinking water for a final concentration of 112.5 mg/mL with *ad libitum* access for 25 hours (Figure 3.24 D). Mice drank on average 2.5 mL, which was equivalent to 225 mg of 3'SL within the 25-hour period. Blood samples were collected by tail vein before (0) after 1 hour and every 3 hours thereafter. Results revealed low, but detectable concentrations of 3'SL in blood, with high variability between groups, which peaked during a 3-hour window between the 19 and 22-hour time points.

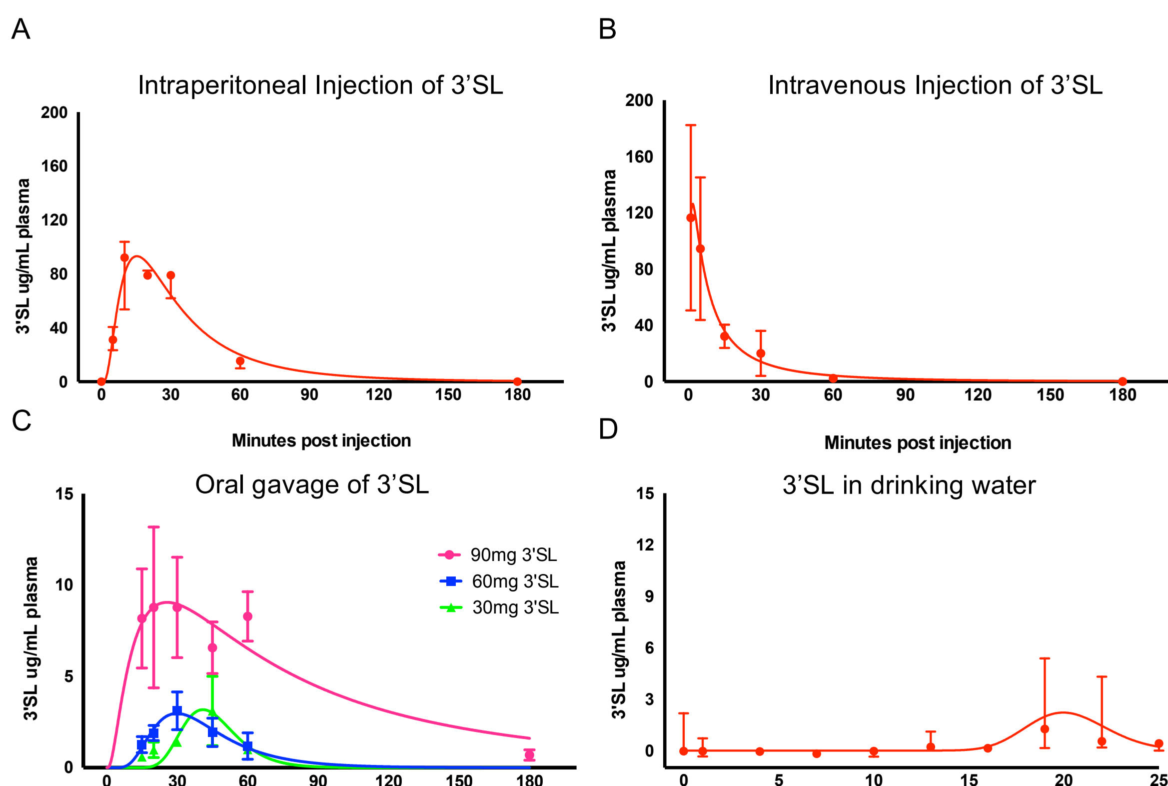


Figure 3.24 Pharmacokinetics of 3'sialyllactose in murine blood varies among intraperitoneal (A), intravenous (B), gavage (C) and oral administration of 3'sialyllactose (D).
Pharmacokinetics of 3'SL concentration in blood plasma before (0 min) and at 15, 20, 30, 60 and 180 min after 3'SL intraperitoneal injection (250 µg of 3'SL) (A). Pharmacokinetics of 3'SL concentration in blood plasma before (0 min) and at 5, 10, 15, 30, 60 and 180 min after 3'SL intravenous injection (100 µg of 3'SL) (B). Pharmacokinetics of 3'SL concentration in blood plasma before (0 min) and at 15, 20, 30, 45, 60 and 180 min after administration by oral gavage (30, 60, 90 mg of 3'SL) (C). Pharmacokinetics of 3'SL concentration in blood plasma before (0 min) and at 1, 4, 7, 10, 13, 16, 19, 22, 25 hours, during oral administration of 3'SL in drinking water (112.5 mg/mL 3'SL) (D). Bars represent the mean ± SEM; n=3-6.

3.7.2 Assessment of 3'sialyllactose efficacy in an *in vivo* murine model for rheumatoid arthritis

Rheumatoid arthritis (RA) is an inflammatory condition depicted by systemic and local inflammation that leads to cartilage and bone destruction and affects nearly 1% of adults globally (Smolen et al., 2016). The cause of RA remains unknown, and its pathogenesis is complex, including many cellular components such as macrophages and cytokines (*IL-6* and *IL-1β*) which are known to perform a significant role in the development and progression of joint destruction. RNA-seq results and *in vitro* experiments revealed the prostaglandin receptor EP4, as a potential major participant in mediating the anti-inflammatory effects of 3'SL in macrophages. Prostaglandins are lipid inflammatory mediators derived from

arachidonic acid via various enzymatic reactions. The binding of PGE_2 to EP4 is complex and plays both beneficial and harmful roles during inflammation (Ricciotti & Fitzgerald, 2011). In addition, RNA-seq results also provided evidence that 3'SL up-regulated genes associated with mediators of pro-resolution in macrophages. Therapies for RA that both inhibit inflammation and activate resolution are still being explored, and hence the *in vivo* effect of 3'SL in a mouse model of RA was investigated.

The mouse model of rheumatoid arthritis known as collagen antibody-induced arthritis (CAIA) which induces arthritis by the systemic administration of LPS in combination with a cocktail of monoclonal antibodies, targeting a major constituent of articular cartilage matrix proteins, known as collagen type II (Chondrex, Inc.). Similarly to human rheumatoid arthritis, the CAIA model results in cartilage degradation and bone erosion due to synovitis with infiltration of polymorphonuclear and mononuclear cells and pannus formation (Matsuo et al., 2016; Perilli et al., 2015). Mice were administered the monoclonal antibody by i.v. injection followed by an intraperitoneal injection of LPS three days later. Immediately following the LPS injection mice were treated with 3'SL or water as a control, which was administered by oral gavage every 8 hours for the duration of the study, until mice were euthanized on day 14 (Figure 3.25).

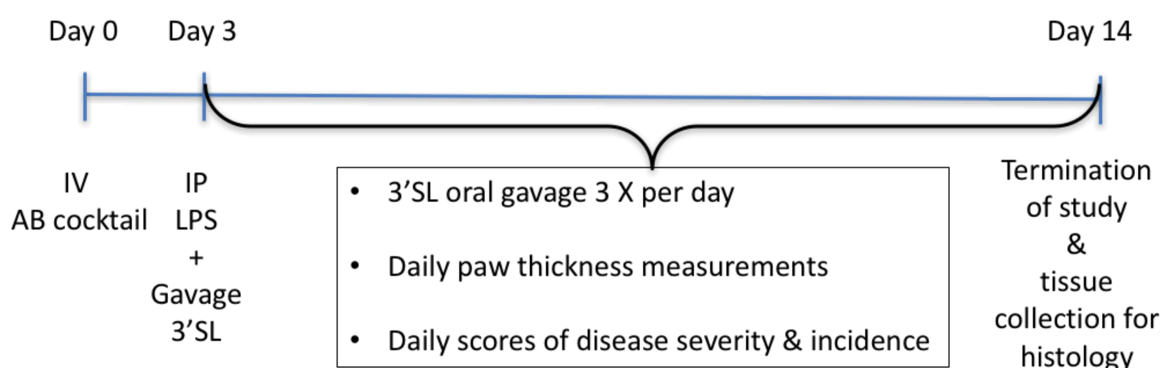


Figure 3.25 Description and timeline of collagen anti-body induced arthritis model. On day 0 a monoclonal antibody (1.5 mg) was administered i.v. followed by 25 ug LPS injection i.p. on day 3. Oral gavage of 3'SL (90 mg) was given every 8 hours for the remainder of the study. Paw measurements and scores measuring incidence and severity were recorded daily. Study was terminated and limbs were collected for histology on day 14.

The development of clinical signs of arthritis such as induced joint inflammation with digital redness and swelling were assessed. Disease activity, severity and inflammation were evaluated daily by a semi-quantitative visual scoring (previously described 2.3.2) and measuring swelling (or ankle thickness) of the affected joints over time. Due to variations in

ankle width among animals, swelling was calculated by comparing the ankle thickness at each time point to day 0, at the onset of disease (Figure 3.26 A) Ankle thickness seemed remarkably less severe in mice fed 3'SL (blue) from day 5 on, however results were only statistically significant by days 13 and 14 ($p < 0.05$). Disease severity (Figure 3.26 B), determined by a maximum clinical index score of 16, was obviously lower in 3'SL treated mice, starting on day 5 and became statistically significant on day 7 ($p < 0.05$). Disease severity significantly improved on days 8, 9 and 10 ($p < 0.01$) and additionally continued through days 11, 12, 13 and 14 ($p < 0.001$). A maximum clinical index score of 28 was used to compare disease incidence in 3'SL treated (blue) and non-treated (red) mice (Figure 3.26 A). By day 5, differences were apparent and deemed significant by day 6 ($p < 0.05$) and day 7 ($p < 0.01$) and continued to rapidly decrease from days 8 through day 14 ($p < 0. 001$).

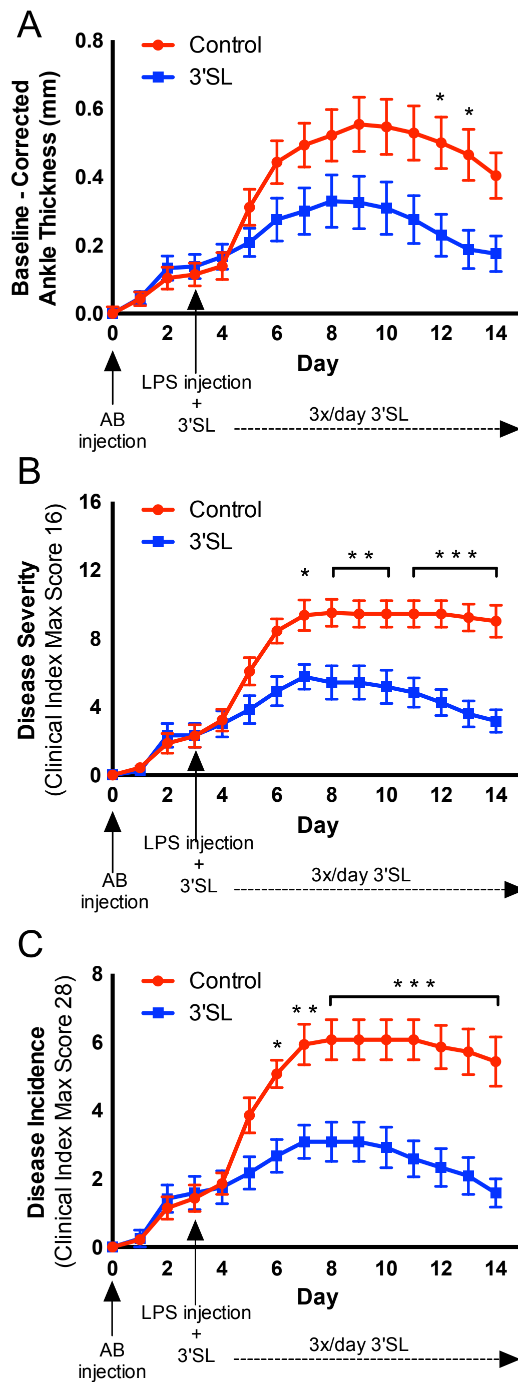


Figure 3.26 Oral administration of 3'sialyllactose reduces signs of inflammation measured by disease severity (A) and incidence (B) in BALB/c mice with collagen antibody-induced arthritis disease.
Induction of arthritis in all mice was achieved by i.v. injection of a monoclonal anti-type II collagen 5-clone antibody cocktail (1.5mg) on day 0 and followed by i.p. injection of LPS from E. Coli 0111:B4 (25 µg) on day 3. Mice were treated with 3'SL (90mg) by oral gavage (n=12), or water as control (n=13) every 8 hours on days 3 through 14. Arthritis was monitored for 14 days. Administration of 3'SL on days 13 and 14 significantly improved severity of arthritis measured by (A) ankle width. However, the clinical index scores for (B) disease severity were significantly less in 3'SL treated mice by day 7. (C) Scores for clinical index of disease incidence we significantly different between groups by day 6, compared to arthritic control animals treated with water. Bars represent mean ± SEM. Statistical differences were assessed analyzed using two-way repeated measures of ANOVA followed by Bonferroni post-hoc test for multiple comparisons. *p < 0.05, **p < 0.01 and ***p < 0.001 was considered significant.

At the termination of the study, on day 14, hind limbs were harvested from all mice and histological evaluations were performed (Figure 3.27 A & B).

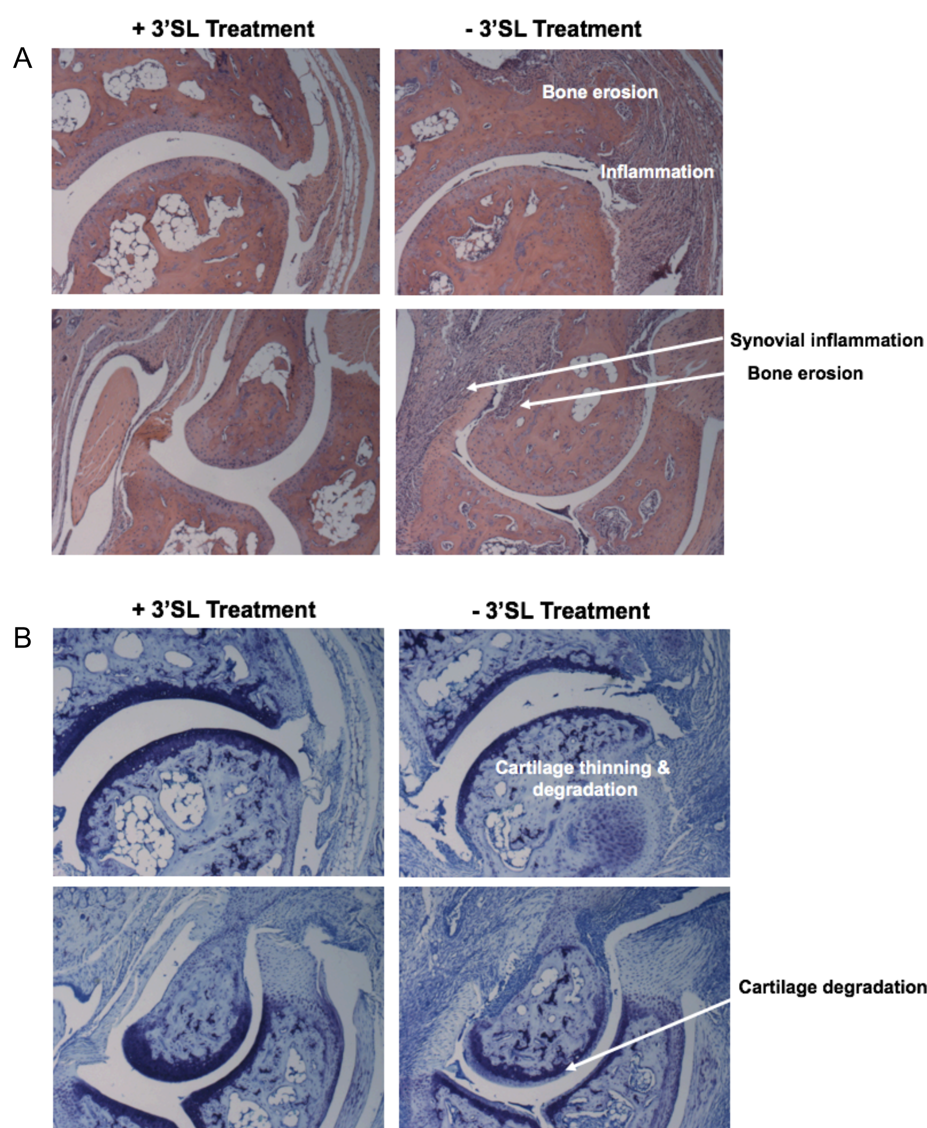


Figure 3.27 Representative histopathology of ankles joints subjected to H&E staining indicating regions of synovial inflammation and bone erosion, (A) and toluidine blue indicating regions of cartilage erosion (B).
Hind limbs from mice fed 3'SL (+3'SL treatment) and mice fed water (-3'SL treatments) were harvested at the end of the study (day 14) for histological evaluation. The arthritis was determined to be aggravated in non-treated control mice when compared to mice treated with 3'SL, by visual assessment of inflammatory infiltrate, bone erosion and cartilage degradation. Representative photomicrographs from 3'SL treated mice (n=2) and non-treated controls (n=2) are shown. Original magnification x50.

Degree of inflammation, bone erosions and cartilage damage were quantified by scoring joint sections on a 0 to 4 scale (previously described 2.3.2). Hematoxylin and eosin stain (H&E) sections of the ankle joints revealed a significant difference in degree of (Figure 3.28 A) inflammation in 3'SL treated mice compared to control group (*p* < 0.01). Furthermore, 3'SL and control mice exhibited a significant difference in the level of (Figure 3.28 B) bone erosion (p < 0.05) and in the amount of (Figure 3.28 C) cartilage damage (p < 0.05). These results indicate that the continuous administration oral of 3'SL reduces the severity and incidence of clinical signs and histological features of the type II collagen induced arthritis in mice.

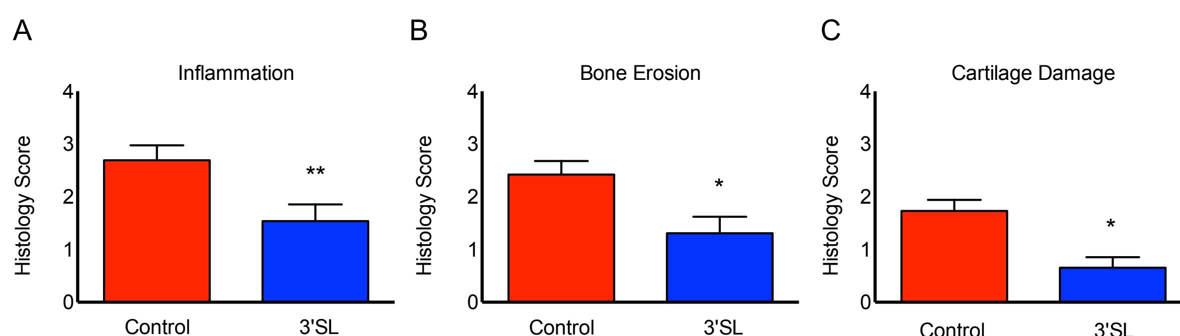


Figure 3.28 Collagen antibody-induced arthritis in BALB/c mice exhibit reduced synovial inflammation (A), bone erosion (B), cartilage damage (C), with oral administration of 3'sialyllactose.
Quantification of the degree of inflammation and bone cartilage degradation, erosion by histological scoring in hind limbs of mice fed 3'SL over time after induction of arthritis. The arthritis was aggravated in non-treated control mice (n=13) as compared to mice treated with 3'SL (n=12). Hind limbs from all mice were harvested at the end of the study (day 14) and prepared for histological evaluation by H&E staining and Safranin-O and scored on a scale of 0-4. Mean ± SEM for each bar is shown, Wilcoxon matched-pairs signed rank test was used, with a criterion of * = p <0.05, ** = p <0.01 for significance.

4 Discussion

Macrophages are thought to play a critical role in the onset and progression of inflammation. They are found in many tissues and exhibit two major functional phenotypes, depending on where they reside. They can demonstrate either pro-inflammatory (classically activated M1) or anti-inflammatory characteristics (alternatively activated M2). Regulatory mediators such as chemokines and cytokines, amongst many others, direct the balance between the two phenotypes within a tissue. A disruption in this balance leads to an increase in M1 phenotype followed by a state of chronic inflammation (Singh et al., 2014). For instance, the accumulation of M1-type macrophages in adipose tissue is associated with obesity-induced insulin resistance and can result in the development of type-2 diabetes mellitus (Odegaard & Chawla, 2008; Schenk et al., 2008). Also, bone marrow macrophages play a central role in chronic inflammation of synovium driving the onset of rheumatoid arthritis; and in atherosclerosis, the accumulation of oxidized LDL in the arterial blood vessels leads to an inflammatory process involving the recruitment of circulating monocytes into the endothelial space (Singh et al., 2014). Additionally, during acute infections such as sepsis, the severity of the disease is related to the induction of M1 macrophage phenotype. *Escherichia coli* (*E. coli*) prompts a typical M1 profile through the recognition of LPS by TLR4 (Pinheiro da Silva et al., 2007) to initiate a systemic inflammatory response, resulting in immune dysregulation and multiple organ failures (Benoit et al., 2008).

The numerous benefits of HMO for breast-fed infants have been explored in a variety of capacities. Studies suggest that they have anti-adhesive properties to protect mucosal surfaces from microbial pathogens, or potential prebiotic effects to influence the gut microbiome, and can serve as immunomodulatory components (previously described in sections 1.1.4 and 1.2.2.1). Additionally, HMO are found undigested in the systemic circulation and in the urine of breast-fed infants, as well as in that of pregnant and lactating women (described in 1.1.3.5). Together, these findings support the approach taken to investigate the effect of HMO on immune cells.

Ongoing research has suggested that HMO aid in lowering the incidence of inflammatory diseases in human breast-fed infants. HMO reduce pro-inflammatory PNC formation and selectin-mediated inflammatory events and dextran conjugated LNnT and LNFP III are thought to drive the expansion of peritoneal suppressor macrophages, resulting in an anti-inflammatory environment (Atochina et al., 2001; Bode, Kunz, et al., 2004; Bode, Rudloff, et al., 2004). Preliminary data in our lab from A. Szyszka's Master's thesis (2014) described the anti-inflammatory effects of pHMO in LPS-stimulated RAW 264.7 macrophages.

To further identify the particular HMO responsible for the anti-inflammatory effects and to characterize the involved mechanisms, both *in vitro* and *in vivo* studies were designed and executed using LPS-stimulated macrophage models. This report describes novel anti-inflammatory mechanisms of 3’SL in reducing the macrophage driven expression and secretions of pro-inflammatory cytokines in various *in vitro* models. The *in vivo* anti-inflammatory effects of 3'SL were further established in a murine model of rheumatoid arthritis. The discovery of 3'SL as a potential macrophage-specific targeted therapy holds promise for both children and adults, in reducing mediators of chronic inflammation, in diseases such as rheumatoid arthritis.

4.1 pHMO and individual HMO attenuate gene expression of inflammatory cytokines in LPS-activated macrophages

4.1.1 Macrophage Models

The experiments in this study were first conducted using an immortalized cell line, known as RAW 264.7 murine macrophages. This cell line was derived from a tumor induced by Albelson murine leukemia virus (Raschke et al., 1978b), and was chosen as a simple model widely used to study inflammatory processes by activation with LPS. To determine that the results are not cell line dependent experiments were repeated in primary macrophages, Bone Marrow Derived Macrophages (BMDM). The majority of *in vitro* experiments were conducted using these cells as the primary model, because they exhibit biological functions and properties of macrophages differentiated from monocytes, and are therefore considered mature, in contrast to macrophage cell lines, which are immature. In addition, BMDM are widely used for genetic screening, functional studies, drug assessment, host-pathogen interactions, and in various other capacities (Trouplin et al., 2013).

Results observed in both Raw 264.7 and BMDM cells, were also confirmed in a human macrophage cell line. Experiments were repeated in THP-1 cells, which are widely used to study immune responses and originate from the peripheral blood of a 1-year old male patient diagnosed with acute monocytic leukemia. In addition, THP-1 macrophages activated by LPS are known to express genes for MD2, CD14, and MyD88, which are essential for LPS signaling *in vivo*, and therefore provide the opportunity to translate the results to a human *in vitro* macrophage model.

4.1.2 Macrophage activation by LPS and inflammatory markers

The commonly used immuno-stimulant LPS is derived from *Escherichia coli* and known to induce inflammatory properties in macrophages. Once TLR4 is bound to its ligand LPS, the

M1-phenotype is induced (Maeshima & Fernandez, 2013). *IL-6* and *IL-1β* are major pro-inflammatory cytokines induced by LPS and were selected as the principal markers of inflammation across most experiments. They are widely studied due to their involvement in many inflammatory diseases. The observed surge in *IL-1β* and *IL-6* gene expression in response to LPS stimulation of macrophages indirectly validated the M1-phenotype and the activation of pro-inflammatory signaling pathways in all of the experiments (Jablonski et al., 2015; Mills et al., 2000). The response in *IL-6* expression to LPS stimulation in macrophages was similar to what had been previously observed. *IL-1β* gene expression also increased due to LPS exposure (Feghali & Wright, 1997; Shimazu et al., 1999; Toshchakov et al., 2002b), and both cytokines decreased in response to the addition of pHMO and particular individual HMO, in all three types of macrophages.

Macrophages cultured in DMEM with only PBS served as a control in every experiment to monitor the base line cytokine expression of non-stimulated cells. The PBS control gave an indirect proof that HMO had no effect on the transcription of the investigated cytokines and cells and experimental reagents were free of LPS or other pro-inflammatory agents. Macrophages were also incubated with DMEM containing only HMO. To minimize the amount of LPS (naturally occurring) in HMO samples, LPS was removed from HMO with endotoxin removing columns.

Several reports have described the induction of TLR4 signaling by specific glycan structures. Kurakevich et al., (2013) demonstrated that mouse pups receiving milk from wild-type dams containing 3’SL were here more likely to develop colitis than pups receiving milk devoid of 3’SL. In addition, the study suggested that 3’SL activated isolated DC of mouse lymph node in a TLR4-dependent manner, and therefore 3’SL was the cause of inflammation. TLR4 activation has been shown to be necessary for the immune priming (Vatanen et al., 2016) and therefore one can speculate that activation of the immune system by HMO via TLR4 may be essential for early immune development. In contrast to what has been previously shown, this study highlights the anti-inflammatory effects of 3'SL in LPS-stimulated macrophages. However, when commercially available 3'SL from different vendors was tested before endotoxin removal, *IL-6* mRNA expression was drastically increased and prevented the anti-inflammatory effects of 3'SL from being apparent (data not shown). These results were misleading, and the effect of 3'SL on macrophages was in fact driven by the presence of LPS traces present in commercially available 3'SL preparations and not by 3'SL itself. Macrophages amongst other immune cells are known to be particularly sensitive to LPS contamination (Schwarz et al., 2014). Insufficient removal of LPS can result in activation of these cells and mask the actual immunomodulatory properties of HMO.

4.1.3 3’SL was identified as the HMO with the most potent anti-inflammatory effects

To establish whether a specific HMO structure was responsible for the anti-inflammatory response in macrophages, a multi-step approach was employed. Distinctions between pooled neutral and acidic HMO were first evaluated in RAW 264.7 cells. Further fractionated neutral and acidic oligosaccharides were tested on macrophages, and HMO identified within the most effective sub-fractions were individually tested, and pro-inflammatory cytokine gene expression was measured as an indicator of effectiveness.

Experiments conducted with pooled HMO divided into two fractions (neutral and acidic) revealed reductions in *IL-1β* and *IL-6* gene expression levels that were comparable in both fractions (Figure 1.9). Sub-fractions of both groups were further analyzed and showed that both neutral and acidic sub-fractions reduced gene expression with some variations (Figure 3.1). Traces of acidic HMO were detected in the neutral fractions 9 and 10. Interestingly, the sub-fractions that were effective in reducing *IL-1β* and *IL-6* gene expression contained 3’SL (Table 3.1). Since the sub-fractions did not include distinct acidic and neutral HMO, chemically synthesized oligosaccharides that were present in the most effective sub-fractions were further tested, based on their commercial availability.

The acidic and neutral sub-fractions reduced gene expression to different degrees. Neutral sub-fractions exhibiting the clearest effects were comprised of DFLNT, LNFP I, LNDFH I or II, and/or LNT. The HMO DSLNT, LST a, b, and c, 3’SL, and/or 6’SL were present in the acidic sub-fractions. Since sub-fractions containing 2’FL were not effective, 2’FL was used as a negative control. The HMO identified in the successful sub-fractions are supported by various reports studying the systematic effects of HMO on immunity. The neutral HMO LNFP II and LNnT are thought to have effects on peritoneal suppressor macrophages (Terrazas et al., 2001), while several other indications show that acidic HMO have various immunomodulatory properties. For example, acidic HMO were discovered to affect leukocyte trafficking and PNC formation (Bode, Kunz, et al., 2004; Bode, Rudloff, et al., 2004). They are also thought to influence lymphocyte maturation and possibly prevent allergic immune responses (Eiwegger et al., 2004, 2010).

Experiments to further elucidate on the functional structure of HMO were performed and revealed that the effects were indeed structure specific (Figure 3.3). The acidic HMO 3'SL, 6'SL and DSLNT all share similar linkages. 3'SL and 6'SL were shown to have an inhibitory effect on the pro-inflammatory cytokine *IL-6,* and are both structurally similar with sialic acid moieties linked to galactose by an α2,3 and α2,6 linkage, respectively. In contrast, the acidic HMO DSLNT is sialylated by an α2,3 linkage to galactose and an α2,6 linkage to the internal GlcNac and showed no anti-inflammatory effects. Since each sialic acid contributes one

negative charge to the HMO, the additional charge from DSLNT may interfere with its binding to the macrophage receptor. To test this hypothesis, LPS-activated macrophages could be exposed to the HMO sialyl-lacto-N-tetraose (LST b), which resembles the structure of DSLNT without an α2,3 sialic acid. In addition, the molecular weight of DSLNT is nearly twice that of 3'SL or 6'SL (1290.14 g/mol vs. 633.55 g/mol). The difference in size and charge of the structure may hinder the steric interactions of DSLNT and the macrophage receptor.

Likewise, the neutral HMO LNFP-1 and 2’FL are both fucosylated by an α1-2 linkage, and yet LNFP-1 demonstrates the ability to reduce *IL-6* in Raw 264.7 cells, while 2’FL has no effects. These differences may be due to the difference in fucose linkages between these two structures. 2’FL is comprised of a lactose backbone with an α1-2 fucose linkage at the terminal Gal. In contrast, LNFP-1 has a lactose backbone that is elongated by a lacto-N-biose structure at the non-reducing end. The fucose linkage of LNFP-1 is at the terminal Gal of lacto-N-biose and may affect its binding affinity to the macrophage receptor. Interestingly, the other HMO (DFLNT, LNDFH I or II) that were present in the most effective neutral sub-fractions also contain an α1-2 fucose linked to an elongated backbone (LNT or LNnT). Further experiments could be conducted to further explore the structure specificity of these HMO to macrophage driven inflammation.

Even though 3'SL, 6'SL, and LNFP-1 are structurally different, they may bind to distinct receptors to induce the similar anti-inflammatory effects, or they may associate with different binding pockets within the same receptor. Interestingly, the anti-inflammatory effects of LNFP-1 in Raw 264.7 cells were not apparent in BMDM. These discrepancies may be attributed to the inherent differences between cell lines and primary cells. Berghaus et al., (2010) amongst others, suggest that RAW 264.7 cells most closely resemble BMDM both in phenotype and function. However, they are not cloned, and alterations in their phenotype and function over the course of continuous culture have been reported. Hence, caution has been advised when deducing outcomes obtained with RAW 264.7 cells, and should, therefore, be compared side-by-side with primary macrophage-lineage cells before making any conclusions. Although we can conclude that the observed effects of HMO on macrophages are structure specific, these results do not provide further insight on which explicit feature of the structure is responsible for decreasing the surge in *IL-6* expression in LPS-stimulated macrophages.

Results indicated that the anti-inflammatory modalities were most prominent in Raw 264.7 macrophages and BMDM exposed to the sialylated HMO 3'SL or 6'SL. Concentrations of 100 µg/mL were used, but are considered higher than physiologically relevant when pertaining to breast-fed infants. If HMO were shown effective at lower physiological concentrations, they

could provide benefits for breast-fed infants receiving HMO in early life through mother's milk, and possibly prevent chronic inflammatory diseases later in adulthood. To mimic the state of HMO present in the systemic circulation and to determine the most effective concentration to use in the following experiments, dose-response relationships were performed between the amount of HMO and the gene expression of inflammatory mediators, *IL-6* and *IL-1β.*

A wide range of 3'SL and 6'SL concentrations (1 ng/mL to 1 mg/mL) were tested, and variations between the HMO concentrations for both 3'SL and 6'SL were observed in BMDM. *L*ow concentrations of 6'SL did not significantly decrease IL-6 and IL-1β gene expression. When BMDM were treated with higher concentrations (100 μg/mL) of 6'SL, both *IL-6* and *IL-1β* were reduced compared to LPS treatment. Although it was not significant, there was a trend in *IL-6* reduction in response to 6’SL which seemed to be dose dependent (Figure 3.5 A and B). In contrast, the highest concentrations 100 μg/mL and 1,000 μg/mL of 3’SL significantly decreased expression levels of *IL-6* and *IL-1β* in a dose dependent manner. There was a reduction in *IL-6* expression by 3'SL that was apparent at 10 μg/mL but was not significant. However, even the lowest concentrations of 3'SL from 1 ng/mL to 10 ng/mL, reduced the gene expression of *IL-1β* (by approximately 20-35%), although results were not statistically significant. Physiological concentrations in infants such as 10 μg/mL, although not significant, had some impact on inflammatory mediators reducing the degree of expression by 35% compared to LPS alone and could suggest that 3'SL provided to breast-fed infants could aid in reducing cytokine expression profiles of macrophages. However, these results also indicate that the administration of higher concentrations of 3'SL may provide a benefit as a natural therapeutic compound to reduce pro-inflammatory mediators of inflammatory diseases. Based off of these experiments the IC_{50} of 3’SL for *IL-6* was determined to be 17 μg/mL and 16 μg/mL for *IL-1β*, indicating that similar concentrations of 3'SL are required to reduce the expression of both cytokines. These results also suggest that 3'SL at concentrations of 100 μg/mL is effective in reducing the expression of *IL-6* and *IL-1β.*

LPS stimulation of macrophages leads to the production of many cytokines and chemokines, and to determine if the effects of 3’SL went beyond that of *IL-6* and *IL-1β*, the inhibition of *CCL2* and *iNOS* were also investigated. Both *iNOS* and the *CCL2* chemokine are up-regulated by pro-inflammatory cytokines and are signature molecules for M1 macrophages. However, they both are thought to play a critical role in immune suppression and the regulation of macrophage polarization (Geming Lu et al., 2015; Sierra-Filardi et al., 2014). Interestingly, results indicate that 3'SL exposure inhibits M1 macrophages, and therefore the ability of 3'SL to promote and favor the M2-like phenotype in macrophages remains inconclusive in these experiments.

4.1.4 3'SL reduces cytokine concentrations in macrophage media after 24 hours

Results confirmed that the inhibitory effects of 3'SL on *IL-6* and *IL-1β* mRNA levels translate to their corresponding protein levels. Since LPS induces the expression of many inflammatory mediators (other and *IL-6* and *IL-1β*) (Nau et al., 2002), a multiplex ELISA was used to detect various cytokines and chemokines that are important in many biological processes, including inflammation response and immune regulation. The assay quantitatively determines concentrations of *IL-6* and *IL-1β* in addition to *IFN-γ, IL-2, IL-4, IL-5, CXCL-1, IL-10, IL-12p70,* and *TNF-α,* in macrophage supernatants. These specific biomarkers are relevant as they are implicated in many conditions, including rheumatoid arthritis, Alzheimer's disease, asthma, atherosclerosis, allergies, obesity, multiple sclerosis, diabetes, and Crohn's disease (reviewed in Moudgil & Choubey, 2011; Santamaria, 2003)

Previous experiments show that 3'SL attenuated the LPS-induced increase of *IL-6* gene expression in macrophages. After a 24-hour incubation period of macrophages with LPS and 3'SL, similar results confirm a translation to the protein level for *IL-6, IL-2, IL-4, IL-5, IFN-γ,* and *IL-12p70.* Nonetheless, the protein production of other cytokines including *IL-1β, IL-10, TNF-α,* and *CXCL-1* was not significantly reduced by 3'SL (Figure 3.8). These findings are of translational importance and support the evidence of 3'SL as an inhibitor of *IL-6* gene mRNA expression and *IL-6* secretions. The effects of 3'SL or lack thereof on these cytokines may provide insight into a mechanistic pathway employed to attenuate macrophage activation by LPS, and help determine if 3'SL can reverse an imbalanced pro-inflammatory immune response towards a balanced response.

Previous experiments show in various LPS-activated macrophage cell models that 3'SL significantly reduces relative *IL-1β* mRNA expression. In this experiment, there was an apparent trend towards 3'SL reducing *IL-1β* secretions compared to LPS alone; however, results were not statistically significant. These results may be attributed to limitations associated with the multiplex assay. Only the 24-hour time points were included in the assay due to the number of wells available on the plate. Since it is known that cytokine secretions are time–dependent, cytokine concentrations may vary within a 24-hour time frame. Higher concentrations of *IL-6* may be more apparent after 24 hours, whereas *IL-1β* concentrations may only peak after 48 hours. In addition, each well measures all ten cytokines, and since *IL-6* concentrations were above the standard curve, samples had to be diluted for most cytokines to be accurately detected within range. The assay revealed low concentrations of *IL-1β* which were below the standard curve detection limit in some samples. This may have affected the accuracy of the output and perhaps led to the observed non-significant results.

IL-10 is considered an anti-inflammatory cytokine, typical for promoting the M2-phenotype in macrophages (Mantovani et al., 2002; Kevin W. Moore et al., 2001; Ouchi et al., 2011). Due to the anti-inflammatory nature of *IL-10*, 3'SL was not expected to reduce its concentration. Previous results from Szyszka (2014) with pHMO demonstrated no effect on reducing or increasing *IL-10* mRNA expression, and therefore confirm the observed effects with 3'SL.

TNF-α is a pro-inflammatory cytokine secreted in response to LPS stimulation of macrophages and has been implicated as a causative factor in several inflammatory diseases (Moller, 2000). 3'SL exposure did not achieve a significant reduction in *TNF*-α secretions, and comparable observations from Szyszka (2014) were described for *TNF*-α mRNA expression when LPS-stimulated macrophages were incubated with pHMO.

Chemokine (C-X-C motif) ligand 1 (CXCL1) is a small cytokine belonging to the CXC chemokine family, and has neutrophil chemoattractant activity, but was not significantly reduced by 3'SL. Although neutrophils are involved in the pathogenesis of various inflammatory disorders, perhaps the anti-inflammatory effects are specific to macrophages and employ a signaling pathway which does not incorporate CXCL1 secretions.

This experiment reveals that 3'SL not only reduces the concentrations of pro-inflammatory cytokines but also those of anti-inflammatory cytokines such as *IL-10*, *IL-4,* and *IL-5*. One explanation for this might be that concentrations of anti-inflammatory cytokines rise in response to the stimulation of inflammatory cytokines. Since 3'SL reduces pro-inflammatory cytokines, there may be no need for anti-inflammatory concentrations to increase in the presence of 3'SL, and therefore what appears to be reduced may in fact not be directly affected by 3'SL.

4.2 Determining a receptor responsible for the anti-inflammatory effects of 3'SL

4.2.1 The attenuation of pro-inflammatory cytokine production in LPS-stimulated BMDM by 3'SL is independent of carbohydrate interactions between 3'SL and LPS

The structural integrity of LPS is governed by three structurally distinct regions including the carbohydrate core, the carbohydrate O-antigen region, and the lipid moiety, known as lipid A located at the tail end (Rietschel et al., 1994). As an isolated agent by itself, this lipid portion of LPS binds to TLR4 and is recognized by macrophages, which then initiates a pro-inflammatory response (Raetz & Whitfield, 2002). The sialic acid portion of 3'SL is known to function as a mediator of cellular interactions involving carbohydrate ligands and their receptors, such as Siglecs (Macauley et al., 2014) and attachment processes involving

carbohydrate-carbohydrate interactions (Seah & Basu, 2008). The anti-inflammatory effects of 3'SL in macrophages stimulated with the lipid component of LPS were assessed to rule out the possibility of a possible glycan-glycan interaction (between the carbohydrate region of LPS and 3'SL). The activation of murine BMDM with lipid A resulted in a marked increase in pro-inflammatory cytokines, and the administration of 3'SL effectively attenuated the increased expression of *IL-6* and *IL-1β.* From this particular experiment, the interactions of 3'SL with the lipid portion of LPS could not be ruled out. The observed anti-inflammatory effects could have been caused by a competitive binding of 3'SL to TLR4, thereby preventing Lipid A from inducing the pro-inflammatory cytokine expression. Additional experiments using RNA Seq were conducted and excluded the potential interaction of 3'SL with TLR4. RNA-seq analysis revealed that thousands of genes were differentially expressed in BMDM in response to LPS activation. In comparison, only a hundred genes were differentially regulated when cells were co-treatment with 3'SL and LPS. These results support the notion that the addition of 3'SL led to a particular alteration in gene expression related to LPS stimulation, confirming that the TLR4 activation initiated by LPS was not hindered by the direct interaction of 3'SL.

4.2.2 3'SL does not affect cytokine expression in exogenous TLR4-mediated *IFN*-β stimulation of macrophages

As another approach, the chronic inflammatory mediator *IFN-β* was tested as exogenous stimulants to induce a reduced type of chronic inflammation and to get a further insight into HMO affected signaling pathways. 3'SL was not effective in reducing cytokine expression and may be due to the fact that in *IFN-β* -activated macrophages *IL-1β* and *IL-6* mRNA expression did not increase to the same extent compared to previous experiments using LPS-stimulated macrophages. TLR4 stimulation of induces a MyD88-independent signaling pathway that leads to the production of *IFN- β* (Huizhi Wang et al., 2008). These results may indicate that the receptor-signaling pathways involving *INF-β* are not affected by 3'SL. The anti-inflammatory effects of 3'SL may be independent of the molecular nature of the TLR4 ligand, and 3'SL may directly be interacting with macrophages through a yet unknown receptor.

4.2.3 The attenuation of pro-inflammatory cytokine production in LPS-stimulated BMDM by 3'SL is not mediated by Siglec-E, Sialoadhesin or BAI-1

The question of how 3'SL induces anti-inflammatory effects remained unsolved, and several experiments were therefore conducted to identify a candidate receptor capable of binding

3’SL. The interactions of 3’SL with sialic acid binding cell surface receptors were therefore investigated.

Siglec-E has been shown to modulate TLR-signaling in murine BMDM driving an anti-inflammatory response (Ando et al., 2008; Boyd et al., 2009) via an intracellular effector domain that activates downstream phosphatases (Crocker et al., 1998; Khatua et al., 2013). Although the Sialoadhesin receptor lacks an intracellular effector domain (Macauley et al. 2014), it has been shown to effectively bind ligands consisting of sialic acid α2-3 bound to Galactose (Blixt et al., 2003).

The BAI-1 receptor on macrophages has been shown to recognize the core oligosaccharide component of LPS and mediates the TLR4 inflammatory pathway (Soumita Das et al., 2011). BAI-1 may also be able to recognize and bind to the 3’SL oligosaccharide to interfere with the effective binding of LPS to BAI-1, thus attenuating the pro-inflammatory response.

BMDM were isolated from mice deficient in either Siglec E, Sialoadhesin, or BAI-1. When activated by LPS and co-stimulated with 3’SL, BMDM showed significantly decreased levels of *IL-6* and *IL-1β* expression, compared to the addition of only LPS. These results were comparable to the observed effects of 3’SL on LPS-stimulated wild type BMDM, indicating that none of these receptors mediate the anti-inflammatory effects of 3’SL.

4.2.4 The anti-inflammatory effects of 3’SL may involve candidate receptors ALX/FPR2 or EP4

Genes for the candidate receptors ALX and EP4 were deemed most interesting based on an in-depth analysis of the RNA sequencing data. The transcriptomes of quiescent BMDM (PBS) and BMDM incubated with either 3'SL or LPS alone, and LPS and 3'SL together were compared. RNA sequencing data were analyzed by clustering genes by their functions and common activated or suppressed transcription factors, and genes significantly differentially expressed 2-fold or greater were used for analysis. Genes were annotated by their biological process, molecular function and cellular component. Ingenuity Pathway Analysis (IPA) predicted the EP4 receptor encoded by the PTGER4 gene as a significantly activated upstream regulator of the differentially expressed genes. The promoter regions of the differentially expressed genes were scanned to identify common transcription factors, and factor-binding sites were also identified to reveal the ALX/FPR2 receptor as a potential candidate for a ligand-receptor pathway activated and/or inhibited by 3’SL.

Many of the eicosanoids such as prostaglandins (PG) and leukotrienes (LT) which are derived from arachidonic acid (AA) (Samuelsson et al., 1987) via the cyclooxygenase (COX)

and lipoxygenase (LOX) pathways play a vital role in mediating inflammation and in initiating its resolution phase. Ligands of the receptors EP4 and ALX/FPR2 are derivatives of the COX and LOX pathways, respectively. Lipid mediators that bind to a G protein-coupled receptor (GPCR), known as ALX/FPR2 function as a “stop signal” for inflammation mediators (Bannenberg et al., 2005; Serhan, 2005; Serhan et al., 2008). In addition, they actively participate in dampening host responses to bring the inflammation to an end (Chiang et al., 2005). EP4 is a receptor for PGE_2, which has been shown to be involved in a wide array of inflammatory responses across various tissues.

Both receptors were targeted with agonist and antagonists and results indicated that neither of the antagonists for both of the receptors attenuated 3’SL mediated inhibition of LPS inflammation. In contrast, the EP4 agonist in the presence of 3’SL in LPS-stimulated BMDM had an increased inhibitory effect on *IL-6* mRNA expression, compared to the agonist alone in LPS-stimulated cells. Since this experiment suggests that 3'SL had no effect on ALX/FPR2 receptor, but may act as an EP4 agonist to reduce pro-inflammatory cytokines, further experiments were conducted where EP4 mRNA expression was silenced in Raw 264.7 cells. Here, a nearly 50% reduction in EP4 mRNA expression was achieved and resulted in a loss of the inhibitory effects previously observed by 3'SL on *IL-6* mRNA expression in LPS-stimulated macrophages.

4.2.5 The anti-inflammatory effects of 3’SL are possibly mediated via an EP4 signaling pathway

These results fit accordingly to previous reports. The abundant expression of EP4 in macrophages was confirmed in mouse macrophages (Akaogi et al., 2004; Ikegami et al., 2001; Pavlovic et al., 2006), cell lines J774.1 and RAW 264.7 (Katsuyama et al., 1998; Khan et al., 2012; Tajima et al., 2008), and human macrophages (Bayston et al., 2003; Cipollone et al., 2005; Iwasaki et al., 2003; Kubo et al., 2004; Meng-Hsing Wu et al., 2005).

The involvement of EP4 in inflammation has been widely described, but is relatively complex and poorly understood. *In vivo* studies demonstrate that bone formation is promoted by EP4, and that activation of this receptor seems to play various beneficial roles in osteoporosis and fracture healing. Other studies using EP4 receptor knockout mice report an increase in impaired fracture healing (Li et al., 2005). The use of EP4 agonists have been investigated as a therapeutic potential to facilitate bone healing (Tanaka et al., 2004) and studies in diabetic rats show an increase in bone mineral content and density (Marui et al., 2006). Although the therapeutic effects EP4 agonists have been described, other studies have reported conflicting results suggesting that EP4 antagonists are potentially beneficial in the

treatment of rheumatoid arthritis. EP4-deficient mice showed decreased inflammation, measured by circulating *IL-6* and serum amyloid A levels, as well as by the reduced incidence and severity of disease in CAIA (McCoy et al., 2002). Macrophages were isolated from these EP4 KO mice and reported to produce significantly less *IL-1β* and *IL-6* than control samples. This study suggests that the exacerbation of rheumatoid arthritis was directly attributed to the reduction of EP4 signaling in macrophages (McCoy et al., 2002). Based off of these different findings it seems that the activation of EP4 can lead to either a downregulation of inflammation or contribute to inflammation in various settings. Analysis of RNA seq results using IPA predicts that EP4 is an activated upstream regular, and it is therefore assumed that 3'SL interacts with EP4 at the cell surface of macrophages and acts as an agonist to exert its anti-inflammatory effects.

Studies have examined the role of EP4 signaling in macrophages *in vitro* and described that the predominant PGE receptor subtype, PGE_2 has a dual effect on macrophages. PGE2 signaling via EP4 stimulates macrophage motility (Tajima et al., 2008) but it has also been shown to inhibit the induced inflammatory response (Cipollone et al., 2005) and suppress the release of inflammatory chemokines in LPS-stimulated human primary macrophages (Takayama et al., 2002). A possible speculation of the signaling pathway employed by 3'SL may resemble that of PGE_2 and is illustrated in Figure 4.1. Upon activation of the EP4 receptor by binding of PGE_2, EP4 agonists, or possibly 3'SL, the EP4 receptor–associated protein (EPRAP) is responsible for mediating the anti-inflammatory actions of EP4. EPRAP binds to the long carboxyl terminal cytoplasmic domain of EP4 and limits stimulus-induced phosphorylation at this site. By doing so, EPRAP prevents degradation of NF-kB p105 protein, which is an important cytoplasmic inhibitor of NF-κB and MEK activation (Minami et al., 2008) and therefore augments p105 stability. The LPS-driven inflammatory processes are thereby attenuated by reducing inflammatory gene transcription dependent on NF-κB and decreasing the production of pro-inflammatory factors. To confirm that 3'SL exerts its effects via this particular signaling pathway, experiments verifying the involvement of ERAP must be conducted, and more importantly, receptor-ligand experiments are required to conclude whether 3'SL directly binds to EP4.

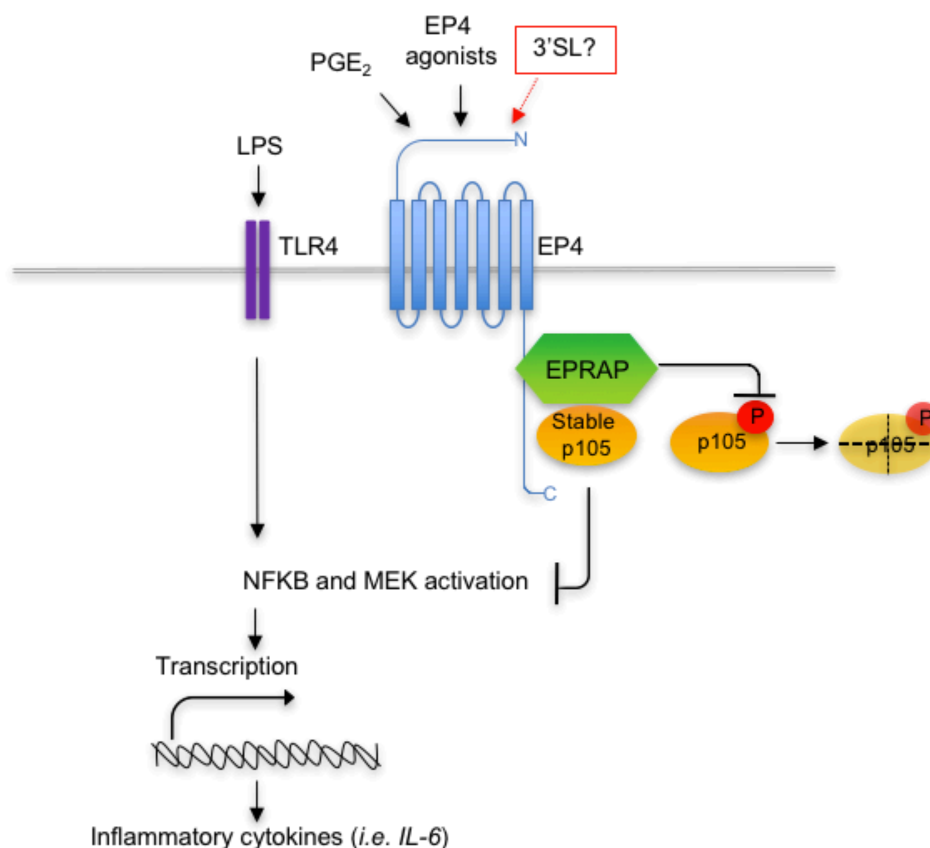


Figure 4.1 Possible signaling pathway employed by 3'SL to exert anti-inflammatory effects in LPS-stimulated macrophages.
PGE_2, EP4 agonist and perhaps 3'SL, promote EPRAP interaction with extended C-terminus of EP4 receptor, to stabilize the p105 subunit by preventing its phosphorylation and degradation. This results in the attenuation of NF-κB and MEK/ERK1/2 activation and further inhibits transcription of pro-inflammatory cytokines in macrophages activated by LPS. Figure adapted and modified from Minami et al., (2008)

4.3 *In vivo* efficacy of 3'SL

4.3.1 Pharmacokinetics of 3'SL

These experiments were conducted to determine the best mode of administration for 3'SL, to maintain 3'SL concentrations high enough to reduce an inflammatory response, and to mimic physiologically relevant concentrations in blood. 3'SL was added to drinking water for 25 hours to determine if a saturation curve would develop with the continuous administration of 3'SL. In this case, concentrations were only apparent for a short 4-hour period, which coincided with the nocturnal tendencies of mice, leading to drinking the largest volumes of

water only at night. In addition, the concentrations observed in blood were much lower than that of the IC_{50} for *IL-6* and *IL-1β* determined in the *in vitro* experiments (Figure 3.5)

3'SL was administered via intravenous and intraperitoneal injections to determine if higher concentrations of 3'SL could be reached in the blood. As expected, the absorption of 3'SL into the blood was greater with these methods of administration compared to the consumption of 3'SL. However, levels in blood declined quickly, and the duration by which 3'SL was detectable was significantly lower compared to oral gavage administration, where a single bolus of 3'SL was given to mice by oral gavage. Concentrations of 3'SL in blood were detectable when mice were administered 3'SL at concentrations of 30, 60, or 90 mg. Blood concentrations of 3'SL were highest and remained apparent for a longer period when mice were gavaged with 90 mg, compared to 30 or 60 mg.

4.3.2 3’SL reduces symptoms of rheumatoid arthritis in an *in vivo* murine model

The autoimmune disease rheumatoid arthritis (RA) is a chronic and systemic inflammatory disorder affecting about 1-3% of the world population (Dieppe, 2002; Sacks et al., 2010). Although several tissues and organs are affected, the flexible (synovial) joints are the primary point of attack, resulting in painful and possible disability. Painkillers and anti-inflammatory drugs are the current treatments used to suppress the symptoms. As the disease progresses, patients receive disease-modifying anti-rheumatic drugs (DMARD) to prevent long-term joint damage by stopping further immune activity. Adverse side effects due to liver toxicity and a lack of immune response leaves the patients taking these drugs susceptible to reoccurring infections (Kahlenberg & Fox, 2011). There is a great need for treatment options aimed at preventing early disease onset and reducing the spread of inflammation to other joints, or the incidence of flare-ups.

The mouse model used for this study, Collagen Antibody-Induced Arthritis (CAIA), was specifically developed after human rheumatoid arthritis. The model was chosen to test 3’SL efficacy *in vivo* because it is dependent on *IL-1β* and easily induced by the injection of a cocktail of monoclonal antibodies directed against type II collagen (C-II), followed by a single injection of LPS. In comparison to other mouse models of RA, the assessment 3'SL was accomplished in a relatively short period (2 weeks vs. 6-8 weeks) and helped minimize the total amount of 3'SL required for this study to achieve significant results.

The pharmacokinetic studies revealed that the oral administration of 90 mg of 3’SL yielded detectable concentrations of 3’SL in blood for up to 3 hours. In this *in vivo* study for rheumatoid arthritis, mice received an oral gavage of 3’SL (90 mg), or water every 8 hours.

BALB/c mice were used for this study because they develop more severe arthritis with higher incidence than C57BL/6 mice. BALB/c mice are thought to be genetically predisposed towards Th2 responses compared to C57BL/6 mice (Reiner & Locksley, 1995). The genetic differences amongst various mouse strains result in variations in the development of the inflammatory phase of the joint disease (Nandakumar et al., 2003). In addition, only male mice were used because there is a known gender and hormone influence on the CAIA model. Estrogen has been shown to exert a suppressive effect on arthritis; therefore males are more susceptible to CAIA than females (Nandakumar et al., 2003)

Clinical scoring was accomplished by awarding a numerical score for each swollen digit, swollen footpad and, or swollen wrist or ankle. These scores were added together to give each animal a total score, reflecting disease incidence or severity. Three to four days after LPS injections, mice receiving 3'SL as at treatment had overall lower scores throughout the remainder of the study. These differences, gradually increased in statistical significance as the study progressed, indicating that 3'SL has the potential to help resolve inflammation by lessening the duration and the gravity of the disease.

Inflammation swelling was measured in the affected joints over time, using a caliper to measure ankle thickness, which assesses the disease activity. Due to the high variability in ankle thickness between animals, a baseline measurement was taken before the study began. Although there was an apparent difference in ankle thickness between 3'SL treated animals and control animals, results only were statistically significant for two days of the study. Ankle thickness in the control group peaked around 9-10 days and steadily decreased over the remaining days. Similar observations were made for the 3'SL treated group, yet ankle thickness measurements always remained lower than that of the control group. The study was terminated on day 14, before ankle thickness in the control group could reach baseline or that of the 3'SL treated group. Histological staining of the inflamed paws was then conducted to determine the effects of 3'SL on synovitis and bone and cartilage erosions. Mice receiving 3'SL as treatment showed variations in inflammation, bone and cartilage erosions, which correlated with clinical scoring observations. For future studies, performing immunohistochemical staining of the cryosections of the arthritis paws could give further insight on immune cell populations and specifically how 3'SL administration affects macrophage infiltrations.

Although the direct effects of 3'SL on macrophages *in vitro* have been established, studies have also shown that 3'SL influences the population of the gut microbiome (Tarr et al., 2015). In addition, imbalances in the microbial community of the gut are related to a multitude of auto-inflammatory and auto-immune diseases and studies have shown a possible role for the

microbiota in the pathogenesis of RA (reviewed in Scher & Abramson, 2011). Further experiments need to be conducted to confirm that the observed results in the mouse model for RA are indeed due to a direct effect of 3’SL that is independent of its effects on the gut microbiome. A similar study of CAIA would have to be repeated where mice would need to be housed individually, and their feces would have to be collected before and during the study on a daily basis. Additional treatment groups of mice would have to be added to the study. Since 3’SL is known to influence the gut microbiome, it would be important to include non-diseased mice 3’SL. In addition, the onset RA may also modify the microbiome, and therefore non-diseased mice receiving the water control would also need to be included. Comparisons in the microbiome between groups would be required to confirm the microbiome-independent effects of 3’SL on reducing the symptoms of RA. Alternatively, studies could be repeated to avoid the GI tract and by-pass the microbiome entirely by altering the method of 3’SL administration (by i.p., i.v, or s.c.).

The administration of the antibody cocktail causes a gradual increase in inflammation that is exacerbated by LPS administration three days later. In this study, the first treatment of 3'SL was given on the same day as LPS. To further elucidate on the mechanistic actions of 3'SL, future studies need to be conducted. The treatment of 3'SL should perhaps begin around the time that inflammation is at its peak, to determine if the effects of 3'SL are still apparent after inflammation has already been initiated. The preventive effects of 3'SL need to be elucidated and feeding 3'SL to mice for a time before the beginning of the study could help determine these effects. In the context of breast-feeding, the study could be conducted in adult mice, which as pups, had been reared on the milk of mice deficient in ST3galIV gene. Without the enzyme to synthesize 3'SL, the mice would have never been exposed to 3'SL and may lead to an increased susceptibility to RA compared to mice reared on normal WT milk.

To determine if the observed effects of 3’SL on RA in mice can also be achieved in human RA patients, a clinical approach should be pursued. Studies have shown that women with RA are likely to go into remission during pregnancy (de Man et al., 2008) and or during lactation (Colebatch & Edwards, 2011; Doran et al., 2004; Jaakkola & Gissler, 2005; Pope et al., 1999). Since each woman’s profile of HMO composition is unique, it would be important to determine if the remission of RA correlates with concentrations of 3’SL in the mother’s milk or blood. Multicenter cohort studies with sites across the world could be set up to recruit thousands of pregnant women suffering from RA. Blood and milk samples would need to be collected from the time of parturition and then extended throughout lactation. A rapid, high-throughput technology would then be used to analyze HMO composition and make associations between concentrations of individual HMO and the occurrence of RA remission. If mothers who underwent remission had significantly higher concentrations of 3’SL in their

milk and blood than their counterparts, then the clinical data would confirm these current preclinical findings. In fact, the prospective longitudinal birth cohort known as the Canadian Healthy Infant Longitudinal Development (CHILD) (Azad et al., 2017) has followed participants over time as they grow and develop—from mid-pregnancy into childhood and adolescence. Breast milk samples and datasets from this cohort have already been collected and will be available for HMO analysis.

The matching results from preclinical studies and independent cohort studies can then help inform the design of a clinical intervention study to prove that 3'SL reduces symptoms of RA symptoms. Many RA patients experience flares during the postpartum period (Barrett et al., 1999). Some medications are compatible with nursing, but in general, the use of medications during lactation can be problematic, and most commonly prescribed RA medications such as Methotrexate and Leflunomide are not safe for the infant and must be avoided (Sammaritano & Bermas, 2014). Infants are already exposed to 3'SL through human milk and therefore 3'SL could potentially act as natural therapeutic to provide relief to breast-feeding patients without harming their infant.

Juvenile RA is known to be more prevalent in infants that are not breast-fed (Mason et al. 1995; Young et al., 2007). To determine if 3'SL in milk lowers the risk of juvenile RA, a longitudinal cohort study could be executed. A number of women and their infants would have to be recruited, based on the incidence of juvenile RA, and their milk would be collected. In addition, the health status of the children would have to be monitored over the course of several years. HMO composition in the mothers' milk would be analyzed to determine associations between concentrations of 3'SL and the occurrence of juvenile RA. Clinical studies such as this one could provide more insight on the potential effects of 3'SL so that it could potentially become a supplement that mothers could add to their milk to prevent the onset of juvenile RA in their children.

4.4 Future directions

The application of 3'SL as an anti-inflammatory compound should also be considered in other fields. Macrophages are found in all tissues and modulate homeostasis and normal physiology through their broad functional diversity (Handschin & Spiegelman, 2008; Wynn et al., 2013). Their regulatory roles are often disrupted, and therefore macrophages contribute to a variety of inflammatory diseases (Figure 4.2).

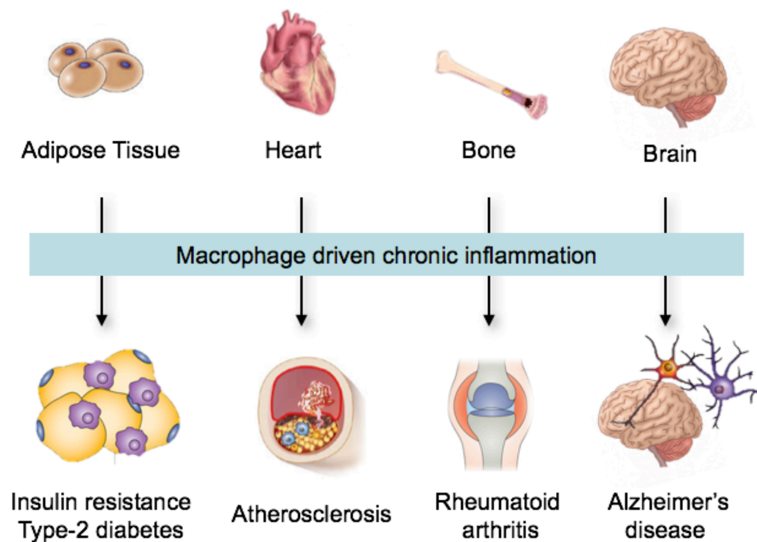


Figure 4.2 Macrophage-driven inflammation in various tissues is linked to the development of chronic diseases.
Examples of tissues and the effects of inflammation are shown. Recruitment of macrophages in adipose tissue are linked to the development of insulin resistance and type 2 diabetes. Accumulation of macrophages in arteries of the heart are associated with atherosclerosis. The local and continuous production of pro-inflammatory cytokines from macrophages in bone result in rheumatoid arthritis. Inflammatory responses in the brain lead to neurodegenerative diseases. (figure adapted and modified from Handschin & Spiegelman, 2008; Wynn et al., 2013).

Data from Figure 3.23 indeed that 3'SL can attenuate oxLDL induced foam cell formation, and supports a role for 3'SL in possibly modulating foam cell conversion, an important contributor to atherosclerosis. The pathogenesis of atherosclerosis is complex and characterized by the accumulation of lipids and inflammatory cells such as macrophages, within the arteries. This process drives thickening and narrowing of the arterial wall, reducing blood flow to many vital organs. Circulating levels of LDL increase and get trapped and modified into oxLDL. Which in turn induce attraction and binding of immune cells including monocytes that differentiate into macrophages and internalize the LDL and oxLDL particles. These macrophages accumulate cholesterol and become lipid droplet-loaded cells known as foam cells. In addition, oxLDL can induce M1 activation by stimulating TLR4 signaling, amplifying the chronic inflammatory response, and this process eventually transitions to more complex advanced plaques with significant clinical consequences. (Lusis, 2000).

Cardiovascular diseases (CVD) are globally the leading cause of death for both men and women, primarily due to atherosclerosis. Despite recent advances, the development of safe and effective therapeutics has yet to be established. In combination with the promising results of 3’SL reducing foam cell conversion, the gene ontology analysis of the RNA

sequencing data revealed that 3'SL stimulation affects genes involved in macrophage cholesterol homeostasis (Figure 3.18). In addition, several studies have reported the implication of macrophage EP4-signaling pathways as potential modulators of atherosclerosis pathogenesis (Babaev et al., 2008; Tang et al., 2011). With this preliminary work and findings, the efficacy of 3'SL as a therapeutic for atherosclerosis is promising, and further studies *in vivo* models should be conducted to elucidate the role of 3'SL in atherosclerosis and the chronic inflammatory state of various other diseases.

4.5 Conclusion

The notion that oligosaccharides in human milk have the ability to modulate the immune system is further strengthened by the results described in this study. Based on the data gained from these experiments, the specific HMO 3'SL can lower intracellular inflammatory responses in primary mouse and human macrophages induced by LPS binding. 3'SL decreased the mRNA expression and protein concentrations of several the pro-inflammatory cytokines in addition to *IL-6* and *IL-1β*. In LPS-stimulated macrophages, the observed effects of 3'SL on mRNA expression of *IL-6* and *IL-1β* were shown to be concentration dependent. In addition, through thorough investigation, the receptor EP4 was discovered to participate in the mechanistic properties by which 3'SL suppresses anti-inflammatory responses in macrophages. Direct binding of 3'SL to the EP4 receptor must be confirmed before identifying EP4 as the responsible structure for the how 3'SL exerts its anti-inflammatory effects on LPS-induced macrophages

This study went beyond the conventional connotation that the biological importance of HMO pertains to only infants. The anti-inflammatory effects of 3'SL were confirmed in an *in vivo* murine model of rheumatoid arthritis. These results substantiate the use of HMO as a potential therapeutic or preventative compound, given in doses higher than physiologically relevant to infants, for the treatment of chronic inflammation in adults. This is the first study that reports the anti-inflammatory effects of 3'SL on macrophages *in vitro*, which can be further translated to rheumatoid arthritis *in vivo*. Whether similar effects of 3'SL are observed in human patients suffering from rheumatoid arthritis has yet to be elucidated. The pre-clinical *in vitro* and *in vivo* studies in combination with future human cohort studies, will help elucidate on the mechanisms of 3'SL in reducing RA symptoms and help enable future intervention studies in adults to determine the effectiveness of ingesting 3'SL in humans suffering from RA

It is important to note that current medications for RA have adverse side-effects. As a result, these treatments are reluctantly prescribed to young children and are not compatible for use

in patients who are pregnant or breast-feeding. The natural occurrence of 3'SL in milk could provide an alternative solution for treating RA in these particular patients. Until recently, clinical research studies have been limited by the insufficient availability of HMO (Bode et al., 2016). Due to advances in HMO isolation, purification and synthesis new opportunities are surfacing for the large-scale production of HMO. For example, bovine milk oligosaccharides isolated from dairy streams are used as precursors for chemical and enzymatic synthesis of structures resembling HMO (reviewed in Bode et al., 2016). Chemical synthesis is also used to build large libraries of HMO with various combinations of structures, lengths, branching and sialylation/fucosylation. HMO studies focused on structure-activity relationship are comparable to current drug discovery approaches and thus greatly benefit from a widespread chemical space of distinct HMO. In addition, bioengineered microbes such as *E. coli* have been constructed to produce 2'FL (Baumgärtner et al., 2013; Won-Heong Lee et al., 2012) and 6'SL (Drouillard et al., 2010). Microbial metabolic engineering is commonly used as a pharmaceutical and food manufacturing process and is currently the most promising approach to generate large quantities of HMO. Overcoming these production hurdles has enabled the use of HMO for clinical trials (Goehring et al., 2016; Marriage et al., 2015) and for commercial application.

The HMO 2'FL and LNnT, both synthesized in bioengineered microbes, have been approved by the United States Food and Drug Administration (FDA) for use as infant food product through the GRAS (Generally Recognized As Safe) pathway. In Europe, these same HMO, have undergone novel food applications with the European Food Safety Authority (EFSA). Overcoming the regulatory obstacles for these specific HMO has paved the way for the approval of 3'SL as a food product in the near future (Bode et al., 2016).

The results presented describe the potent inhibitory effects of 3'SL on macrophage-driven inflammation and provide first evidence for the use of 3'SL as a promising natural anti-inflammatory compound. Recent advances in the synthesis of individual HMO have led to the large-scale production of 3'SL and will help drive intervention studies and clinical trials. Once the regulatory hurdles have been overcome, such studies will generate additional evidence to help translate the scientific promise of 3'SL towards a commercial opportunity; thus providing a variety of possible therapeutic applications to treat children, pregnant or lactating mothers and many others suffering from RA and potentially other chronic inflammatory diseases.

Supplemental

Supplemental 1 Summary of genes significantly down-regulated when BMDM are exposed to 3’SL, compared to PBS alone.
Cells were stimulated for 6h with 3’SL (100 µg/mL). Cells cultured in DMEM with PBS and without 3’SL, served as control. n=3

Symbol	Description	Fold change	p-value
Card11	caspase recruitment domain family, member 11	4.070	0.003
Prodh	proline dehydrogenase	2.390	0.096
dnmt3aos	DNA methyltransferase 3A	2.336	0.092
Top1mt	DNA topoisomerase 1, mitochondrial	2.203	0.089
Fads6	fatty acid desaturase domain family, member 6	2.199	0.064
Fabp4	fatty acid binding protein 4, adipocyte	2.133	0.022
Ankrd37	ankyrin repeat domain 37	2.002	0.006

Supplemental 2 Summary of genes significantly up-regulated when BMDM are exposed to 3’SL, compared to PBS alone.
Cells were for 6h with 3’SL (100 µg/mL). Cells cultured in DMEM with PBS and without 3’SL, served as control. n=3

Symbol	Description	Fold change	p-value
gm20658	predicted gene 20658	4.330	0.048
Ednrb	endothelin receptor type B	4.246	0.006
Pde4d	phosphodiesterase 4D, cAMP specific	3.751	0.016
Acss2	acyl-CoA synthetase short-chain family member 2	3.497	0.089
Lrp8	low density lipoprotein receptor-related protein 8	3.389	0.017
Cytip	cytohesin 1 interacting protein	3.385	0.001
atp2b4	ATPase, Ca++ transporting, plasma membrane 4	3.230	0.002
Panx1	pannexin 1	3.214	0.040
Gpr141	G protein-coupled receptor 141	3.139	0.006
osgin1	oxidative stress induced growth inhibitor 1	3.051	0.089
mturn	RIKEN cDNA 2410066E13 gene	2.970	0.026
Prkar2b	protein kinase, cAMP dependent regulatory, type II beta	2.633	0.097
ahr	aryl-hydrocarbon receptor	2.625	0.002
Tanc2	tetratricopeptide, ankyrin repeat, coiled-coil containing 2	2.592	0.076
ahrr	aryl-hydrocarbon receptor repressor	2.591	0.002
Tshz1	teashirt zinc finger family member 1	2.530	0.089
Cbr3	carbonyl reductase 3	2.509	0.085
Gab1	growth factor receptor bound protein 2-associated protein 1	2.477	0.000
Tspoap1	benzodiazapine receptor associated protein 1	2.437	0.097
Edil3	EGF-like repeats and discoidin I-like domains 3	2.378	0.071
tlr5	toll-like receptor 5	2.362	0.055
GRK5	G protein-coupled receptor kinase 5	2.351	0.071
Odc1	ornithine decarboxylase	2.347	0.026
pde3b	similar to phosphodiesterase 3B	2.321	0.035
Tsku	tsukushin	2.227	0.058
Trim16	F-box WD-40 domain protein 10; tripartite motif-containing 16	2.164	0.076
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	2.127	0.047
Cas1	catalase	2.122	0.089
Tfec	transcription factor EC	2.116	0.031

Trib3	tribbles homolog 3 (Drosophila)	2.113	0.071
Myom1	myomesin 1	2.099	0.064
Mmp19	matrix metallopeptidase 19	2.077	0.006
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	2.076	0.025

Supplemental 3 Summary of genes significantly down-regulated when BMDM are exposed to LPS and 3'SL, compared to LPS alone.
Cells were activated with 10 ng/mL LPS and stimulated simultaneously for 6h with 3'SL (100 µg/mL). Cells cultured in DMEM with LPS and without 3'SL, served as control. n=3

Symbol	Description	Fold Change	p-value
Cxcl3	Chemokine (C-X-C motif) ligand 3	4.37	<0.001
IL6	Interleukin 6	4.12	<0.001
GFI1	Growth factor independent 1	4.03	0.01
TNFSF15	Tumor necrosis factor (ligand) superfamily, member	3.92	<0.001
Tmem178	Transmembrane protein 178	3.38	0.011
CSF3	Colony stimulating factor 3 (granulocyte)	3.27	0.018
Saa3	Serum amyloid A 3	3.14	<0.001
PTGS2	Prostaglandin-endoperoxide synthase 2	3.05	<0.001
IL23R	Interleukin 23 receptor	2.87	0.061
F10	Coagulation factor X	2.8	<0.001
FABp3	Fatty acid binding protein 3	2.65	<0.001
H2-M2	Histocompatibility 2, M region locus 2	2.64	<0.001
Adora2a	Adenosine A2a receptor	2.63	<0.001
CXCL2	Chemokine (C-X-C motif) ligand 2	2.62	<0.001
IL1α	Interleukin 1 alpha	2.62	0.004
CD200	CD200 antigen; similar to MRC OX-2 antigen homolog	2.48	<0.001
CXCL1	Chemokine (C-X-C motif) ligand 1	2.47	<0.001
2310014L17Rik	RIKEN cDNA 2310014L17 gene	2.43	0.037
Zfp558	Zinc finger protein 558	2.37	0.013
Shisa3	Shisa homolog 3 (Xenopus laevis)	2.35	<0.001
RASGRP1	RAS guanyl releasing protein 1	2.35	<0.001
TRIM36	Tripartite motif-containing 36	2.33	0.003
Egr1	Early growth response 1	2.3	<0.001
RAB11FIP1	RAB11 family interacting protein 1 (class I)	2.29	0.002
FABp3-ps1	Fatty acid binding protein 3, pseudogene 1	2.27	0.024
AW112010	Expressed sequence AW112010	2.25	0.035
BCKDHB	Branched chain ketoacid dehydrogenase E1	2.18	0.01
Zfp658	Zinc finger protein 658	2.17	<0.001
Fam102b	Family with sequence similarity 102, member B	2.16	<0.001
CP	Ceruloplasmin	2.15	0.056
Susd2	Sushi domain containing 2	2.14	0.072
Zfp811	Zinc finger protein 811	2.13	0.087
Clic5	Chloride intracellular channel 5	2.12	<0.001
Expl	Extracellular proteinase inhibitor	2.11	0.038
Tagap1	T-cell activation GTPase activating protein 1	2.09	<0.001
CCL3	Chemokine (C-C motif) ligand 3	2.07	0.001
Clec4e	C-type lectin domain family 4, member e	2.02	<0.001
IL12B	Interleukin 12b	2.01	0.006
Hivep3	Human immunodeficiency virus type I enhancer	2.01	0.016
CCRL2	Chemokine (C-C motif) receptor-like 2	2.01	<0.001

Supplemental 4 Summary of genes significantly up-regulated when BMDM are exposed to LPS and 3'SL, compared to LPS alone.

Cells were activated with 10 ng/mL LPS and stimulated simultaneously for 6h with 3'SL (100 µg/mL). Cells cultured in DMEM with LPS and without 3'SL, served as control. n=3

Symbol	Description	Fold Change	p-value
LRP8	low density lipoprotein receptor-related protein 8	2.97	<0.001
BTBD11	BTB (POZ) domain containing 11	2.58	0.054
SLC40a1	Solute carrier family 40 (iron-regulated transporter) member 1	2.41	<0.001
KCNK13	Potassium channel, subfamily K, member 13	2.22	<0.001
6430548M08Rik	RIKEN cDNA 6430548M08 gene	2.16	0.026
Gpr183	G protein-coupled receptor 18	2.08	0.097
CREB3L2	cAMP responsive element binding protein 3-like 2	2.03	0.003
EEPD1	Endonuclease/exonuclease/phosphatase family domain cont.1	2.03	0.003

Supplemental 5 Summary of activated upstream regulators of genes differentially expressed when BMDM are exposed to LPS and 3'SL, compared to LPS alone.

Upstream Regulator	Log Ratio	Activation z-score	p-value of overlap
PTGER4	-0.153	3.403	1.32E-16
U0126		3.367	4.53E-08
epigallocatechin-gallate		3.068	3.62E-10
SB203580		2.997	1.30E-07
Nr1h		2.752	2.18E-08
IL10	-0.09	2.699	1.24E-07
DUSP1	0.036	2.664	4.66E-11
SP600125		2.653	5.54E-07
ABCA1	-0.112	2.64	6.15E-11
miR-155-5p		2.584	4.47E-06
PD98059		2.51	2.13E-05
FBXO32	-0.116	2.438	2.41E-07
NR1H2	0.13	2.407	6.24E-09
15-deoxy-delta-12,14 -PGJ 2		2.405	4.04E-05
RPSA	0.218	2.401	5.68E-09
PRDM1	-0.01	2.401	1.68E-05
Bay 11-7082		2.382	8.90E-08
curcumin		2.363	1.51E-03
VIP		2.342	2.59E-08
N-acetyl-L-cysteine		2.241	2.58E-07
cyclomaltodextrin		2.236	3.95E-09
ABCG1	-0.207	2.235	6.37E-10
resveratrol		2.23	4.78E-06
pyrrolidine dithiocarbamate		2.229	4.07E-05
Alpha catenin		2.224	8.91E-05
nifedipine		2.219	1.08E-06
GW3965		2.217	1.95E-06

apigenin		2.216	5.28E-06
CORT		2.207	5.63E-08
IL37		2.202	6.37E-10
PPARG	0.401	2.2	7.33E-07
IL1RN	-0.385	2.187	2.21E-05
SIGIRR		2.186	2.32E-09
diphenyleneiodonium		2.183	2.24E-06
ZFP36	0.035	2.182	9.76E-08
Sb202190		2.182	5.02E-05
H89		2.182	3.27E-05
sirolimus		2.133	3.21E-02
butyric acid		2.034	1.39E-02
infliximab		2	3.49E-05
rabeprazole		2	3.33E-08
MEOX2		2	1.31E-05

Supplemental 6 Summary of inhibited upstream regulators of genes differentially expressed when BMDM are exposed to LPS and 3'SL, compared to LPS alone.

Upstream Regulator	Log Ratio	Activation z-score	p-value of overlap
lipopolysaccharide		-4.18	2.6E-10
APP	0.003	-3.89	3.0E-11
IL1B	-1.007	-3.72	5.7E-09
TNF	-0.971	-3.71	7.9E-09
Salmonella serotype lipopolysaccharide		-3.67	2.0E-15
poly rI:rC-RNA		-3.64	3.3E-10
MYD88	-0.235	-3.63	1.1E-13
IFNG		-3.49	4.8E-12
TLR3	-0.252	-3.38	2.6E-11
CEBPB	-0.563	-3.36	1.7E-10
CSF2		-3.32	2.8E-08
TLR9	0.21	-3.25	3.0E-12
peptidoglycan		-3.25	1.8E-14
trinitrobenzenesulfonic acid		-3.25	5.1E-15
NFkB (complex)		-3.24	2.4E-08
IKBKB	-0.174	-3.23	3.2E-09
TLR4	0.151	-3.23	1.6E-08
Pam3-Cys-Ser-Lys4		-3.10	1.0E-12
E. coli B5 lipopolysaccharide		-3.10	2.8E-08
E. coli B4 lipopolysaccharide		-2.98	9.6E-07
uric acid		-2.96	5.6E-11
zymosan		-2.96	1.6E-13
salmonella R595 lipopolysaccharides		-2.96	5.4E-11
CpG ODN 1668		-2.93	3.8E-13
IL1		-2.92	1.2E-08
phorbol myristate acetate		-2.92	8.0E-05
MALP-2s		-2.92	2.7E-15

AGT		-2.90	1.2E-05
CpG oligonucleotide		-2.89	7.4E-10
P38 MAPK		-2.84	1.4E-07
TLR2	-0.524	-2.82	9.8E-09
IL1A	-1.39	-2.81	1.2E-08
NOD2	-0.451	-2.80	2.2E-10
N-acetylmuramyl-L-alanyl-D-isoglutamine		-2.79	3.2E-13
RELA	-0.267	-2.79	1.3E-07
IL17A		-2.78	1.4E-07
Tnf (family)		-2.77	1.1E-08
CD40LG		-2.77	4.3E-06
ERK1/2		-2.72	1.5E-09
Immunoglobulin		-2.68	8.5E-08
5-O-mycolyl-β-araf-(1-2)-5-O-mycolyl-α-araf-(1-1')-glycerol		-2.65	7.5E-08
PTPRJ	-0.106	-2.65	4.5E-09
cigarette smoke		-2.62	9.3E-07
CHUK	0.116	-2.60	5.8E-08
Jnk		-2.60	2.5E-06
MIF	-0.29	-2.59	1.3E-09
TLR5		-2.59	1.5E-11
Ap1		-2.58	1.9E-07
TICAM1	0.011	-2.58	3.2E-10
enterotoxin B		-2.58	8.5E-09
LEP		-2.56	1.2E-08
resiquimod		-2.54	4.0E-11
IL2		-2.46	1.6E-06
SAMSN1	-0.622	-2.45	6.3E-06
KRT17		-2.45	1.4E-09
TREM1	0.592	-2.44	2.3E-10
IL22		-2.43	2.7E-08
palmitic acid		-2.43	9.0E-07
C5AR1	0.4	-2.43	9.8E-10
CpG ODN 1826		-2.42	5.5E-08
Ca2+		-2.42	1.1E-05
IL18	-0.337	-2.42	2.1E-06
RAF1	-0.031	-2.42	1.2E-04
TLR7	-0.433	-2.41	7.0E-07
dextran sulfate		-2.41	1.4E-08
F2		-2.40	5.0E-04
CEBPD		-2.40	1.3E-08
LCN2	-0.429	-2.40	1.9E-07
TNFSF12	-0.25	-2.39	3.5E-07
CREB1	-0.221	-2.39	1.4E-03
carrageenan		-2.39	9.2E-09
KITLG	0.176	-2.39	4.5E-05
IL6	-2.042	-2.38	2.4E-07

IL33	0.09	-2.38	1.0E-07
E. coli serotype 0127B8 lipopolysaccharide		-2.38	6.0E-05
TAC1		-2.36	6.5E-08
TBK1	-0.147	-2.35	3.0E-11
STAT3	-0.151	-2.35	2.0E-08
hydrogen peroxide		-2.32	3.1E-04
AGER		-2.31	1.2E-07
CCL5	-0.758	-2.31	7.8E-07
PPP2CA	0.028	-2.24	2.0E-07
PRKCA	0.094	-2.23	3.0E-05
Ccl2	-0.406	-2.23	6.6E-07
C5		-2.23	3.0E-08
5-hydroxytryptamine		-2.23	1.7E-06
1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine		-2.23	4.2E-06
CCR5		-2.23	2.5E-07
RIPK2	-0.198	-2.22	1.6E-05
reactive oxygen species		-2.22	2.1E-06
PLG		-2.22	1.3E-07
D-fructose		-2.22	7.8E-07
tributyrin		-2.22	7.3E-06
PRKCE	0.033	-2.22	6.6E-06
RORC		-2.21	2.9E-08
CXCL2	-1.307	-2.21	9.9E-09
Pkc(s)		-2.20	2.7E-04
HRAS	0.012	-2.20	1.3E-03
camptothecin		-2.20	1.2E-03
CLEC7A	0.179	-2.20	6.4E-09
IL17F		-2.20	1.5E-08
PTGS2	-1.608	-2.20	8.3E-06
CD14	0.138	-2.19	3.2E-08
HMGB1		-2.19	2.2E-06
FABP4	-0.372	-2.19	4.2E-08
lipoteichoic acid		-2.19	7.3E-06
EP300	0.108	-2.19	3.6E-06
Tlr		-2.19	1.4E-05
E. coli lipopolysaccharide		-2.18	5.9E-06
doxorubicin		-2.18	1.6E-02
EGR1	-1.202	-2.18	5.4E-05
ADIPOQ		-2.18	8.9E-05
ozone		-2.18	7.5E-08
leukotriene D4		-2.18	3.3E-06
Pam3-Cys		-2.17	6.6E-07
bleomycin		-2.16	3.0E-05
lysophosphatidic acid		-2.16	1.4E-05
dinoprost		-2.16	1.3E-05
HSPD1	-0.102	-2.16	2.1E-08

imiquimod		-2.15	5.3E-06
VEGFA	0.142	-2.15	2.2E-03
CXCL12	0.067	-2.14	4.3E-04
cyclic AMP		-2.14	4.2E-04
TNFSF11		-2.14	2.4E-07
JAK2	-0.27	-2.13	4.3E-06
TNFRSF1A	0.137	-2.13	2.1E-09
MAPK8	-0.093	-2.10	1.5E-04
SPP1	-0.278	-2.08	1.5E-08
NFKB1	-0.32	-2.05	5.8E-08
lipid A		-2.05	4.6E-08
Ige		-2.03	2.0E-12
ERBB2		-2.03	1.6E-04
Vegf		-2.01	1.2E-04
MARK2	-0.059	-2.00	6.5E-06
SASH1	-0.062	-2.00	6.0E-04
DOCK8	0.052	-2.00	4.8E-04
nitrofurantoin		-2.00	3.8E-04
Pka		-2.00	3.7E-04
3M-001		-2.00	2.8E-06
SOCS6	0.031	-2.00	2.2E-06
misoprostol		-2.00	2.0E-07

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Declaration of Academic Integrity

I certify that the presented work is, to the best of my knowledge and belief, original and the result of my own investigation. Utilized published information is properly and duly acknowledged. The work has not been submitted, either in part or whole, for a degree at this or any other university.

Erklärung

Ich erkläre an Eides statt, dass ich die bei der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

The Therapeutic Potential of Human Milk Oligosaccharides in the Context of Chronic Inflammation

Lehrstuhl für Ernährungsphysiologie

unter der Anleitung und Betreuung durch: Prof. Dr. Hannelore Daniel ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Ab. 6 und 7 Satz 2 angebotenen Hilfsmittel benutzt habe.

Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt. Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt. Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

San Diego, CA. USA, October, 2017

Chloe Augustine Autran

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17. Bazanella M, Maier VT, Clavel T, Lagkouvardos I, Lucio M, Maldonado-Gòmez MX, **Autran CA**, Walter J, Bode L, Schmitt-Kopplin P, Haller D (2017) Randomized controlled trial on the impact of early life intervention with bifidobacteria on the healthy infant fecal microbiota and metabolome. (Submitted)

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