The Effect of *Lactobacillus rhamnosus* GR-1 Supernatant on Endotoxin-Induced Cytokine Secretion in Human Myometrial Cells

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Physiology
University of Toronto

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2017

ABSTRACT

Prophylactic administration of probiotic *Lactobacillus rhamnosus* *GR-1* supernatant (GR1SN) was shown to prevent lipopolysaccharide (LPS)-induced preterm birth (PTB) in mice by suppressing pro-inflammatory cytokine expression in uterine tissues *in vivo*. The aim of this thesis is to elucidate the effects of GR1SN in human myometrial cells *in vitro*, where I hypothesize it will suppress LPS-induced cytokine secretion. Here I demonstrate that PBS-based GR1SN contains protein moieties that induce endotoxin tolerance-like phenotypes in myometrial cells to suppress secretion of pro-inflammatory factors following LPS stimulus. The observed effects are unique to a GR1SN-myometrial cell interaction, and cannot be replicated using supernatant derived from non-*GR-1* lactobacilli species. However, *in vivo*, GR1SN-PBS does not delay the onset of LPS-induced PTB in pregnant CD-1 mice under the experimental conditions used. Through this project, I have highlighted the unique capacity of protein moieties secreted by *L. rhamnosus GR-1* to inhibit inflammatory signals in human myometrial cells.
ACKNOWLEDGEMENTS

Writing this thesis has not only provided me the opportunity to summarize the work I did through my master’s project but also reflect on the people that have motivated me through it all.

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Finally, an endless thanks to my mum. Thank you for all your patience, your understanding, and your support in the choices I have made during my master’s project.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BN</td>
<td>Benzonase Nuclease</td>
</tr>
<tr>
<td>BV</td>
<td>Bacterial Vaginosis</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned Media</td>
</tr>
<tr>
<td>COX</td>
<td>Cycloxygenase</td>
</tr>
<tr>
<td>CST</td>
<td>Community State Type</td>
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<tr>
<td>CXCL</td>
<td>Chemokine (CXC motif) Ligand</td>
</tr>
<tr>
<td>DMEM/F-12</td>
<td>Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony Stimulating Factor</td>
</tr>
<tr>
<td>GD</td>
<td>Gestational Day</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
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<tr>
<td>GR1SN</td>
<td>\textit{L. rhamnosus} GR-1 supernatant</td>
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<td>GR1SN-MRS</td>
<td>MRS-based GR1SN</td>
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<td>GR1SN-PBS</td>
<td>PBS-based GR1SN</td>
</tr>
<tr>
<td>hBD-3</td>
<td>Human (\beta)-Defensin-3</td>
</tr>
<tr>
<td>hTERT-HM</td>
<td>Human Myometrial Smooth Muscle Cell Line Immortalized with Human Telomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ITS</td>
<td>Insulin-Transferrin-Selenium</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine Growth Restriction</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus Kinase and Signal Transducers and Activators of Transcription</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td>Lactobacillus rhamnosus</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipotoichteic acid</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>MAMP</td>
<td>Microbe-Associated Molecular Pattern</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemoattractant Protein-1</td>
</tr>
<tr>
<td>MD2</td>
<td>Lymphocyte Antigen 96</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin Light Chain</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MRS</td>
<td>de Man, Rogosa and Sharpe</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>OXTR</td>
<td>Oxytocin Receptor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-Associated Molecular Pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>Prostaglandin F2α</td>
</tr>
<tr>
<td>pH</td>
<td>Power of Hydrogen</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>pPROM</td>
<td>Preterm Premature Rupture of Membranes</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen Recognition Receptor</td>
</tr>
<tr>
<td>PS80</td>
<td>Polysorbate 80</td>
</tr>
<tr>
<td>PTB</td>
<td>Preterm Birth</td>
</tr>
<tr>
<td>PTL</td>
<td>Preterm Labour</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum Free Media</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of Cytokine Signalling</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Buffer, Acetic Acid, EDTA</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNIL</td>
<td>Term Not-in-Labour</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TOLLIP</td>
<td>Toll Interacting Protein</td>
</tr>
</tbody>
</table>
CHAPTER 1: LITERATURE REVIEW

1.1 Preterm labour: an overview

Preterm delivery, birth of an infant prior to 37 weeks gestation, is a global medical problem that affects millions of mothers and infants annually. Thirty percent of these cases are medically induced to alleviate predisposing gestational complications such as preeclampsia or intrauterine growth restriction (IUGR) (Agrawal and Hirsch, 2012), but other cases of preterm labor (PTL) are idiopathic and occur spontaneously. One known cause of spontaneous PTL (sPTL) is intrauterine infection, found in 40% of all preterm births (PTB) (Agrawal and Hirsch, 2012). Additionally, correlations have been observed between PTB and maternal stress, shortened cervix, uterine insufficiencies, uterine over-distension, and preterm premature rupture of fetal membranes (pPROM), (Romero et al., 2014a) making the treatment of PTB challenging to target.

Current therapeutic interventions for PTB include prostaglandin (PG) synthesis inhibitors (indomethacin) and calcium channel blockers (nifedipine) as more common tocolytics, as well as oxytocin receptor (OXTR) antagonists (atubiban), magnesium sulfate, or nitric oxide donors as less commonly used options. However, these drugs are all associated with high maternal and fetal side effects, and do not target the underlying cause that initiates the premature contractions (Simhan and Caritis, 2017). The use of antibiotics as adjunct therapy for women with urogenital tract infections and intact membranes has been reviewed in a 2013 meta-analysis, indicating that though antibiotics result in reduced infection, they are ineffective at preventing or delaying PTL (Flenady et al., 2013). Interestingly, recent studies have indicated an association between vaginal dysbiosis and infectious PTL, suggesting a protective role of the vaginal microbiome when in homeostasis, offering a potential target for future clinical interventions. Today, the main goal of
tocolytic treatment is to delay labour sufficiently to allow the administration of antenatal drugs like corticosteroids to improve neonatal outcome. As such, current medical advances have focused on the survival of the preterm neonate, which are costly and require technologies that are limited to urban centres, leaving a large proportion of those affected by PTB in developing countries unable to gain access to proper treatment. To achieve global equality in healthcare and improve current management of PTB, novel therapeutic methods to address PTL are urgently required.

1.2 Inflammation and labour

Over the past decade, research has demonstrated that maternal inflammation is a key regulatory event that precipitates both pathological and physiological labour. In term labour, stimulants such as functional progesterone withdrawal, uterine stretch, and fetal and maternal endocrine stimulation activate local inflammatory processes which involve production of cytokines and chemokines which function in autocrine, paracrine, or endocrine mechanisms to recruit peripheral leukocytes, (Shynlova et al., 2012, 2013a, b) and activate local transcription factors such as NF-kB and AP-1. These pathways increase production of labour-associated molecules like matrix metalloproteinases (MMP), prostaglandins (PG), oxytocin, and gap junction proteins like connexin-43 (Cx-43) that induce cervical softening and dilation, fetal membrane rupture, and synchronized myometrial contractions to expel the fetus from the uterus. In cases of pathological PTL, these same pathways can be activated upon host recognition of pathogens Escherichia coli, Ureaplasma urealyticum, Mycoplasma hominis, and Streptococcus agalactiae (Peltier et al., 2010). The inflammatory pathways that orchestrate labour are comprised of more than 50 chemokines that instigate local migration of maternal peripheral blood leukocytes into uterine tissue (Mackay, 2001). Leukocytes include five subtypes; namely
neutrophils, eosinophils, basophils, monocytes and lymphocytes. Following activation, neutrophils become degranulated and trigger angiogenesis or angiostasis (Salamonsen et al., 2002), and monocytes differentiate into macrophages, which phagocytose apoptotic cellular debris or pathogens. All leukocytes promote further production of chemokines and attract more leukocytes to amplify the inflammatory response, explaining the difficulty in reversing labour after its initiation. Although the complete spectrum of stimulants that activate maternal inflammation prior to labour has yet to be elucidated, recognition of pathogens within the genital tract by host pathogen recognition receptors (PRRs) like toll-like receptors (TLRs) play an important role in inducing infectious PTL.

1.3 Toll-like Receptors

TLRs are a transmembrane family of PRR proteins that recognize various endogenous and microbial ligands (i.e. microbial-associated molecular patterns – MAMP) to activate immune responses in host cells. Often, they function as homo- or hetero-dimers to activate downstream pathways and transcription factors such as NF-κB, which regulate expression of inflammatory cytokines and chemokines. In humans, 10 TLRs have been detected. TLRs 1, 2, and 6 function as membrane bound TLR1/2 or TLR2/6 heterodimers to recognize tri- or di-acylated lipoproteins of bacteria. TLR3 is localized intracellularly to recognize viral double stranded (ds)RNA such as PolyI:C. TLR4 is a potent receptor for lipopolysaccharide (LPS), a component of gram-negative bacteria wall, while TLR5 recognizes bacterial protein flagellin. TLR7 and TLR8 serve as receptors for single stranded (ss)RNA, and TLR9 recognizes unmethylated CpG DNA intracellularly. TLR10 agonists remain unknown to date.
1.3.1 Toll-like receptors and labour

TLRs are associated with term and preterm labour, intrauterine infections, and pregnancy complications through activation of immune responses in gestational tissue. In the human cervix, an increased expression of TLR2 and TLR4 with decreased expression of TLR3 and TLR5 are thought to be responsible in part for cervical remodelling at labour (Ekman-Ordeberg and Dubicke, 2012). Decidual cells also express TLR1, TLR4, and TLR6 contributing to infection-associated PTB (Patni et al., 2007). A recent study suggested alterations in tissue sensitivity induced by epigenetic modifications that are associated with increased TLR2 and TLR9 expression in decidual cells isolated from women with spontaneous PTL (sPTL) (Walsh et al., 2017). In the placenta, TLR2 and TLR4 have been detected on trophoblast cells, while choriocarcinoma cells respond to exogenous ligands for TLR2, 3, 4 and 9 (Patni et al., 2007). Interestingly, in placentae of preterm deliveries, the functional activity of TLRs characterized by cytokine production was decreased, although TLR gene expression remained unaltered as compared to term controls (Patni et al., 2015), indicating various alterations in both expression and function of TLRs in relevance to labour. Amniotic epithelial cells also express TLR2 and TLR4, which is significantly increased in term labouring samples as compared to term not in labour. In the human myometrium, TLR2 and TLR4 are increased during term labour compared to preterm, and TLR2 is highly expressed during labour than non-labouring states. (Youssef et al., 2009)

The importance of TLR4 in regulation of parturition has been demonstrated in animal studies, where TLR4 KO mice deliver on average 13 hours later than wild type controls, and newborn pups show increased perinatal mortality. These mice demonstrate decreased expressions of pro-inflammatory cytokines, namely interleukin (IL)-1β, IL-6, IL-12β, and tumor necrosis factor (TNF)-α in placental and fetal membrane tissues when compared to control mice.
Moreover, a TLR4 polymorphism (Asp299Gly) that is associated with impaired TLR4 function in humans has been detected in higher prevalence in preterm infants when compared to infants born at term. (Lorenz et al., 2002) These studies help outline the contribution of TLRs, especially TLR4 to labour, and identified them as potential targets in therapeutic intervention for prevention or treatment of PTB.

1.4 Cytokines, Chemokines and Labour

The activation of TLRs stimulates multiple downstream pathways that result in expression of inflammatory cytokines, which are small extracellular signaling proteins including interleukins (IL), interferons (IFN), and growth factors. Secreted by host cells, cytokines function in auto-, para- or endocrine fashion by binding to target receptors. Pro-inflammatory cytokines moderate the classical phenotypes of inflammation such as vascular dilation, increased endothelial permeability, and leukocyte activation that regulate cellular apoptosis or pathogen removal, while anti-inflammatory cytokines counteract these events to protect the inflamed tissue. Although moderate levels of inflammation alleviate infection and promote pathogen removal, prolonged inflammation can lead to excessive cellular apoptosis and cause tissue damage. Near term, pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 (Winkler et al., 2001; Kemp et al., 2002) and labour-mediating molecules like MMPs or PGs augment cervical dilation, membrane rupture and myometrial contraction. Cytokine concentrations vary in a tissue specific manner; the myometrium expresses increased concentrations of TNF-α, IL-1β, and IL-6 during term labour and LPS-induced PTL, while PTL induced by progesterone antagonist mifepristone demonstrated increased IL-1β and IL-6 expression. (Shynlova et al., 2013a) The choriodecidua shows increased levels of CCL2, 4, 5, 8, and 10 in women prior to PTL, (Hamilton et al., 2013) and increased expression of IL-8, IL-1β, IL-6, and TNF-α is
detected in the cervix (Patni et al., 2007) and amniotic fluid. (Holst et al., 2011) Interestingly, pro-inflammatory cytokines such as IL-1β, IL-6, and IL-8 are also increased in the maternal plasma, (Torbé et al., 2007) indicating a systemic response to local signals.

1.4.1 Pro-inflammatory cytokines

1.4.1.1 Tumour necrosis factor alpha (TNF-α)

TNF-α is a major acute phase inflammatory cytokine, that is expressed by most cells. Immune cells such as lymphocytes, macrophages, NK cells, neutrophils, mast cells, and eosinophils produce both secreted and membrane-bound forms of TNF-α, which allow intercellular communication to induce inflammation, cellular apoptosis, or proliferation. (Christiaens et al., 2008; Kawano et al., 2012) During pregnancy, tight regulation of TNF-α expression is required as unprecedented elevations of TNF-α have been associated with increased risk of fetal loss, gestational diabetes, hypertensive syndromes such as preeclampsia, and IUGR. (Brogin Moreli et al., 2012) During healthy pregnancies, low concentrations of TNF-α are maintained in gestational tissue until a rapid increase during labour, (Hayashi et al., 2008; Shynlova et al., 2013a) which stimulates prostaglandin and MMPs production in the cervix to induce cervical ripening and in placental and myometrial tissues to induce membrane rupture and myometrial contraction. (Christiaens et al., 2008)

1.4.1.2 Interleukin 1-beta (IL-1β)

IL-1β is an upstream regulator of inflammation that stimulates multiple downstream pathways. In pregnancy, increased IL-1β expression has been detected in cervico-vaginal tissue approaching labour (Heng et al., 2014) and shown to induce production of MMP, promoting cervical ripening and dilation, while decreasing production of TIMP (endogenous inhibitors of MMPs). (Patni et al., 2007) COX-2, a rate-limiting enzyme involved in prostaglandin synthesis,
is also further induced by IL-1β. PGE$_2$, in association with cytokines TNF-α and IL-1β are powerful promoters of uterine contraction in the myometrium. High IL-1β production is also detected in fetal amniochorion and decidua in both term and preterm labour regulating membrane rupture. In the myometrium, IL-1β is also responsible for increased oxytocin secretion, leading to increased concentrations of intracellular calcium. The consequence of these events are powerful synchronized myometrial contractions, which expel the fetus.

Experiments in pregnant rhesus monkeys demonstrated that intra-amniotic infusions of TNF-α and IL-1β can induce PTB within 1.1 days from point of injection, compared to 27.6 days in control monkeys (Sadowsky et al., 2006). It was observed that the administration of TNF-α and IL-1β induced production of IL-8, IL-6, PGs, and MMP-9 in amniotic fluid, resulting in an amplified inflammatory response and leukocyte infiltration (Sadowsky et al., 2006). In contrast, treatment with IL-6 or IL-8 did not induce preterm myometrial contractions (treatment to delivery interval was 21.9 days) or increased TNF-α and IL-1β expression in pregnant rhesus monkeys, demonstrating an upstream regulation of IL-6 or IL-8 by TNF-α and IL-1β infusion. Knock-out experiments have further confirmed these findings as pregnant mice with TNF-α, IL-1β double receptor knockouts were protected against local bacteria-induced PTL with reduced myometrial production of PG synthesizing enzyme COX-2 (Hirsch et al., 2006), while IL-6 /-- mouse did not show resistance to bacteria-induced PTL. (Yoshimura and Hirsch, 2003)

1.4.1.3 Interleukin-6 (IL-6)

IL-6 is an inflammatory cytokine secreted by immune cells to induce an acute phase inflammatory response along with TNF-α, and IL-1β. IL-6 stimulates differentiation and maturation of immune cells such as B- and T-cells, as well as hematopoiesis in the bone marrow (Robertson et al., 2010). In gestation, IL-6 is a critical element for the timely onset of parturition,
regulating genes involved in PG-mediated uterine activation, (Papatheodorou et al., 2013) as indicated by delayed labour in term IL-6 knockout mice as compared to wild type mice (Robertson et al., 2010). No associations between IL-6 expression and gestational age have been found; although increased IL-6 mRNA and protein expression has been characterized in the laboring myometrium. (Elliott et al., 2000; Sharp et al., 2016) Specifically, IL-6 expression, along with IL-1β and IL-8 is significantly higher in the lower uterine segment during term labour as compared to PTL, but not in the upper segment. (Tattersall et al., 2008) IL-6 expression was increased in fetal membrane, cervical, and decidual tissue during term and preterm labour with and without infection, indicating its involvement independent of cause of labour onset. (Patni et al., 2007) IL-6 induces OTXR expression on myometrial cell surface (Rauk et al., 2001) although it has no direct effect on myometrial contraction or PG synthesis (Peltier, 2003).

1.4.2 Anti-inflammatory Cytokines

Anti-inflammatory cytokines function to support healthy pregnancies by playing a key role in maintaining inflammatory homeostasis. These include cytokines such as IL-4, IL-10, and IL-13, which are produced by immune cells and certain tissue types to respond to the effects of pro-inflammatory cytokines (Wilczyński, 2005; Romero et al., 2006).

1.4.2.1 Interleukin-4 (IL-4)

IL-4 is an anti-inflammatory cytokine that antagonizes production of pro-inflammatory cytokines TNF-α, IL-1β and PGE₂ by human macrophages. (Hart et al., 1991) In gestational tissue, IL-4 is produced by decidual cells, fetal membranes, cytotrophoblasts as well as maternal and fetal endothelial cells. IL-4 expression increases in gestational tissues such as the trophoblast, decidua and amnion throughout pregnancy (Sykes et al., 2012), suggesting a role in pregnancy
maintenance. However, IL-4 levels do not correlate to fetal development in mice as IL-4 knockout mice do not show impaired fetal growth or development as compared to wild type controls (Svensson et al., 2001).

1.4.2.2 Interleukin-10 (IL-10)

IL-10, is a potent anti-inflammatory cytokine, known to influence the JAK/STAT pathway and inhibit NF-kB activation, to suppress expression of TNF-α, IL-1, IL-6, and IL-12 by human macrophages (Wang et al., 1995; Riley et al., 1999; Saraiva and O’Garra, 2010). Expressed by placental villous trophoblasts and decidual immune cells (Warner et al., 2013), IL-10 is highly produced in 1st and 2nd trimesters, but wanes in the 3rd trimester and prior to labour (Simpson et al., 1998; Hanna et al., 2000). Although IL-10/- mice do not demonstrate impairments to fetal development (Svensson et al., 2001), an increased placental size has been observed in affected animals (Roberts et al., 2003). These mice also show amplified immune response to low dose LPS and un-methylated CpG dinucleotides – ligands for TLR4 and TLR9 respectively (Murphy et al., 2005; Thaxton et al., 2009).

1.4.2.3 Interleukin-13 (IL-13)

IL-13 has close sequence and functional homology with IL-4 (Mak, 2006) to exert anti-inflammatory responses by host cells. In the gut, IL-13 mediates environments which are unfavorable for the survival of pathogens by inducing secretions of molecules such as glycoprotein from gut epithelial cells which detach and remove microorganisms. (Seyfizadeh et al., 2015) Additionally, in human amnion-derived WISH cells, IL-13 inhibits cytokine-induced IL-8 and PGE₂ production. (Keelan et al., 1998)
1.4.3 Colony-Stimulating Factors

M-SCF (CSF1), GM-CSF (CSF2), and G-CSF (CSF3) are multifunctional hematopoietic cytokines that regulate proliferation, differentiation, and survival of macrophages and neutrophils. M-CSF in gestation is involved in early stages through receptor interaction in placental trophoblasts to support fetal implantation and placental development. (Fixe and Praloran, 1997) Furthermore, increased M-CSF in 1st trimester maternal serum is associated with trisomy 21 and maternal gestational diabetes, whereas decreased M-CSF has been associated with trisomy 13, trisomy 18, PTB, and placental insufficiencies. (Eckmann-Scholz et al., 2016) GM-CSF, while bearing inflammatory capacities to enhance granulocyte and macrophage proliferation and maturation, is also recognized as an anti-inflammatory agent that can influence dendritic cell signaling to enhance regulatory T-cell localization and function (Bhattacharya et al., 2015). In mice, GM-CSF has also shown to exhibit neuroprotective effects against neurotoxin MPTP, rendering mice less vulnerable to neurodegeneration in Parkinson’s Disease (Kosloski et al., 2013). In a prospective study, increased GM-CSF expression in cervico-vaginal fluid prior to 24 weeks gestation was detected in association with shortened cervix and PTB (Chandiramani et al., 2012). G-CSF, administered to monocytes, exhibits anti-inflammatory characteristics through suppression of LPS-induced cytokine secretions of IL-1β, TNF-α, IL-12 and IL-18 through JAK/STAT mediated pathways (Boneberg and Hartung, 2002). G-CSF expression is also increased during labour in cervical tissue (Sennström et al., 2000), and in laboring human myometrium (Shynlova et al., 2012). Recent in vitro studies have also demonstrated increased production of GM-CSF and G-CSF in human myometrial cells following static stretch. (Lee et al., 2014)
1.4.4 Chemokines

1.4.4.1 Interleukin-8 (IL-8)
IL-8 (CXCL8) is a major chemoattractant for neutrophils; it is expressed by leukocytes, fibroblasts, endothelial cells and epithelial cells in response to TLR stimulation as a crucial moderator of the innate immune response. IL-8 functions to induce phagocytic activity in activated neutrophils, and to induce target cell exocytosis to secrete histamines, which further induces leukocyte migration (Mak, 2006). In pregnancy, IL-8 expression increases with labour in the cervix, (Sennström et al., 2000) fetal membranes, (Young et al., 2002) and myometrium; (Elliott et al., 2000) secretion of MMP-8 and neutrophil elastase is induced by cervical neutrophils. IL-8 administration to amniotic cells in vitro (but not in chorionic cells) induces MMP-9 expression (Arechavaleta-Velasco et al., 2002). IL-8 expression does not fluctuate in cervicovaginal fluid between sPTB and term laboring samples amongst asymptomatic women with cervical insufficiency (Yoo et al., 2017), although increased IL-8 in cervical fluid has been detected in women with preterm pPROM and intra-amniotic infection (Kacerovsky et al., 2014). High levels of maternal plasma IL-8 have also been characterized in women with severe pre-eclampsia and in women who miscarry in the 2nd trimester, (Arikan et al., 2012) indicating an overall unfavourable physiological function of IL-8 in the maintenance of a healthy pregnancy. Furthermore, high maternal plasma IL-8 in the 2nd trimester has been associated with increased offspring risk of developing schizophrenia in adulthood (Zhang et al., 2004).

1.4.4.2 Monocyte Chemoattractant Protein-1 (MCP-1)
MCP-1 (also known as CCL2) is a monocyte chemoattractant secreted by monocytes, endothelial cells and intrauterine tissues (Mak, 2006). MCP-1 triggers the differentiation of monocytes to macrophages, and stimulates the expression of pro-inflammatory cytokines (Elliott et al., 2000). Its expression is increased in term human myometrium in labour as compared to not-in-labour,
(Sean Esplin et al., 2005) and in the cervix. In women who deliver preterm without infection, high levels of MCP-1 have also been detected in the cervix (Törnblom et al., 2005). In the amniotic fluid, high levels of MCP-1 are found both in the presence and absence of intra-amniotic infection (Esplin et al., 2005).

1.4.5 Other mediators of labour
Inflammatory pathways and cytokines result in activation and increased production of various molecules that are specifically essential to uterine activation in the onset of parturition. These include PGs and their receptors, OXTR, and connexins, which help synchronous contraction of the myometrium. PGs are lipid moieties that are derived from arachidonic acids and converted into active forms by COX-1 or COX-2 as PGH₂, PGI₂, PGE₂, PGF₂α, or PGD₂. Systemically, PGs can induce vasodilation, bronchodilation or bronchoconstriction, and gastrointestinal smooth muscle contraction or relaxation. PGs in the cervix induce local MMP-9 production, which induces cervical remodelling processes. (Ekman-Ordeberg and Dubicke, 2012) In the uterus, increased mRNA expression of COX-2/PTGS2 is detected at term, in both lower and upper uterine segments. (Tattersall et al., 2008) Furthermore, exogenous administration of PGE₂ and PGF₂α have been shown both in vivo and in vitro to induce myometrial contractions, indicating a crucial role of PGs in the regulation of parturition. (Olson, 2003) One mechanism by which PGs induce myometrial contractions along with oxytocin is increased intracellular concentrations of calcium. Both oxytocin and PGs expressions are stimulated by TNF-α and IL-1β, while IL-6 helps increase the expression of OXTR on cell surfaces. Oxytocin also helps to further increase the production of PGs, supporting a strong positive-feedback loop.
1.5 Characterization of vaginal microbiota in non-pregnant women

Ravel et al. defined five community state types (CST) into which healthy vaginal microbiomes were classified based on the bacterial composition. (Ravel et al., 2011) Correlated to this classification were vaginal pH, and Nugent score – a classical diagnostic scoring system observing the shape of rod-shaped bacteria (large, gram-positive scoring 0-4; small, gram-variable scoring 0-4; and curved- gram-variable scoring 0-2). A score of 7 or higher from a total of 10 can indicate bacterial vaginosis (BV), the most common disease of vaginal dysbiosis presented in women of reproductive age. CST-I, II, III and V, dominated by lactobacillus species L. crispatus, L. gasseri, L. iners, L. jensenii respectively, maintain relatively healthier states and have low pH and low Nugent scores, representing low risk for pathogen acquisition. Stability of vaginal health is associated with the dominance of lactobacillus species, which are gram-positive bacteria. Contrastingly, women with CST-IV classification have higher pH, higher Nugent scores, and a variety of anaerobes that inhabit the microbiome with a relatively lower presence of lactobacillus species. Individuals characterized to CST-IV have increased risk of acquiring sexually transmitted diseases and gynecological complications such as BV or vaginal candidiasis. (Romero et al., 2014b)

Although classifying the vaginal microbiome of women help identify those who have an increased risk of acquiring diseases of vaginal dysbiosis, routine events such as the menstrual cycle and sexual activity cause changes in vaginal microbiome, promoting fluidity between CSTs, most commonly between CST-I (L. crispatus) and CST-III (L. iners). (Ravel et al., 2011) The precise role of these species in contribution to vaginal health has been debated as their presence has also been detected in CST-IV microbiomes. Recent studies have demonstrated associations between L. iners and vaginal inflammation and further clinical diagnoses of vaginal
dysbiosis syndromes. (Kindinger et al., 2017) Dominance of L. iners has also been associated with short cervixes and preterm birth <34 weeks. (Kindinger et al., 2017)

To understand such discrepancies and to elucidate the overall functional mechanisms of lactobacilli, scientists have studied their secreted components. Most widely considered of the lactobacilli secretion is lactic acid, which maintains a low vaginal pH that is unfavorable for proliferation of pathogenic species. Lactic acid is found in two isomeric forms – L- and D-lactic acid. L-lactic acid is expressed by both the vaginal epithelium and commensal lactobacilli, while D-lactic acid is only produced by bacterial species. However, the amount of D-lactate production is variable between lactobacilli species, which may explain discrepancies in the protective functions of different lactobacilli. Studies indicate that women whose microbiomes are dominated by L. crispatus present higher concentrations of D-lactic acid than those dominated by L. iners, or pathogenic Gardnerella species, and that L. iners species are incapable of producing D-lactic acid (Witkin et al., 2013).

1.6 Characterization of vaginal microbiota in pregnant women

Lactobacilli colonization is supported by metabolites of mucosal glycogen, which increase with high levels of estrogen. (Spear et al., 2014) Thus, lactobacilli are absent from vaginal flora of pre-pubescent and post-menopausal women, and are more abundant and stable during pregnancies as compared to non-pregnant states. The vaginal microbiome of pregnant women demonstrates augmented presence of L. vaginalis, L. crispatus, L. gasseri, and L. gensenii, and lower presence of bacteria commonly associated with CST-IV. However, women categorized in CST-IV, lack this abundance of lactobacilli and face an increased risk of complications like BV during pregnancy. While some studies have reported no associations between the bacterial composition of a vaginal microbiome and the rates of PTB, (Romero et al.,
others have found an association between higher abundance of pathogenic bacteria in vaginal microbiome of pregnant women with previous history of PTB and who then go on to deliver preterm (Callahan et al., 2017). Some have indicated that a diagnosis of BV during pregnancy can increase a woman’s risk of delivering PTB by up to two-fold (Guaschino et al., 2006). Attempts to treat BV and other complications of vaginal dysbiosis with antibiotics in pregnant and non-pregnant women, are largely insufficient as they only demonstrate success in eradicating the pathogens temporarily but fail to prevent its recurrence. Instead, clinical studies have demonstrated adjunct use of oral lactobacilli probiotics with antibiotics in non-pregnant women help restore homeostasis of the vaginal microbiome to a more stable state, strengthening the health of the woman (Anukam et al., 2009). One such study used a combination of two commensal vaginal lactobacilli species, L. rhamnosus GR-1 and L. reuteri RC-14, which has been found to persist in the vaginal microbiome for up to 19 days following intra-vaginal administration, (Gardiner et al., 2002) to improve the cure and recurrence rate of BV (Anukam et al., 2006).

1.7 Probiotics

Probiotics, which are "live microorganisms which when administered in adequate amounts, confer a health benefit on the host" (FAO and WHO, 2001), have demonstrated no adverse effects when used during pregnancy, and are deemed safe and acceptable. (Dugoua et al., 2009; Lindsay et al., 2014) As such, probiotic Lactobacillus rhamnosus GR-1 supernatant (GR1SN) was used in a recent in vivo mouse study to show its preventative effect to delay the onset of LPS-induced PTB (Yang et al., 2014). The same study found reduced expression of various inflammatory cytokines and chemokines in gestational tissue and peripheral maternal blood, indicating potential mechanisms behind the phenomenon.
1.7.1 Beneficial effects of probiotic administration

To date, most work examining the effects of lactobacillus administration on TLR regulation has been conducted in context of the gut. In the gut, lactobacilli administration appears to result in an improved immune stability, and resilience to endotoxin challenge. Examination of TLR expression and function in Peyer’s patches following oral administration of live L. casei in mice that also received salmonella challenge show an increased expression of TLR2, 4, and 9 as well as reduced TNF-α production. (Castillo et al., 2011) Similar effects were also observed when secondary intestinal epithelial cells (HT-29) were exposed to L. acidophilus in vitro. Cells treated with the probiotic demonstrated a resistance to salmonella-induced increases in TLR2 and TLR4 expressions. (Moshiri et al., 2017) Furthermore, a study which examined the effects of live and heat-killed L. plantarum administration in vitro to secondary intestinal cells (Caco-2) reported that lactobacilli impaired TLR4 mediated NF-kB signalling pathways, as evidenced by increased expression of inhibitory molecules of the pathway such as TOLLIP, SOCS1, and SOCS3. (Chiu et al., 2013) However, such effects remain highly specific for bacterial species and host cell interaction. In an in vivo mouse study where live bacteria or supernatant of L. rhamnosus GG were prophylactically administered by oral gavage prior to radiation to induce apoptosis in crypt cells of the small intestine, a protective effect of lactobacilli was ablated in MyD88-/-, TLR2-/-, and COX2-/- mice, but unaffected in TLR4-/- which implies mechanisms that function independent of TLR4. (Ciorba et al., 2012) In a recent study, live bacteria or GR-1SN has been shown to inhibit hypertrophy in neonatal rat ventricular cardiomyocytes induced by phenylephrine, an α1-adrenergic receptor agonist, (Ettinger et al., 2017) while L. acidophilus promoted growth of epithelial and myofibroblast cells in chicken embryo organoids cultured in vitro. (Pierzchalska et al., 2017) Lactobacilli components such as isolated exopolysaccharides from L. rhamnosus GG have also been injected into mouse fat pads to show improved lipid
metabolism, evident through decreased triacylglyceral accumulation and absence of inflammation. Interestingly, this effect was abrogated in TLR2-/- mice. (Zhang et al., 2016)

In vitro work examining the role of Lactobacilli in mouse macrophages and human monocytic cell lines demonstrated an increase in IL-10 expression. (Kim et al., 2006; Martins et al., 2011) Furthermore, G-CSF expression and secretion have also been shown to increase with Lactobacilli exposure, which affects immune cells in a paracrine manner to inhibit TNF-a production. (Kim et al., 2006) L. rhamnosus administration in malnourished mice demonstrating depleted cytokine production, restored their immune response to control levels, and showed increased levels of IL-10 production above control levels. (Kolling et al., 2015)

In clinical studies, patients on dialysis treatment receiving oral Lactobacilli have demonstrated increased IL-10 expression, (Wang et al., 2015) potentially offering protection from common incidences of inflammation observed in dialysis patients.

Despite the extensive amount of work that has been conducted to examine the role of lactobacilli in the gut, examination of their role in the maintenance of labour remain limited. In vitro studies examining human placental trophoblast cells pre-treated with GR1SN prior to LPS stimulus demonstrated TNF-α suppression and increased IL-10 secretions as compared to LPS control. (Yeganegi et al., 2009, 2011) Chemical inhibition of JAK and p38 revealed the dependency of GR1SN induced IL-10 secretions on JAK/STAT and MAPK pathways. IL-4 levels were not altered following GR1SN treatment in vitro in human trophoblasts, nor in in vivo mouse experiments, remaining consistently high between groups with inflammatory insult and Lactobacilli treatment. (Yeganegi et al., 2009, 2011; Yang et al., 2014) Similar suppression of LPS-induced cytokine secretions by GR1SN were observed in in vitro decidual cultures. (Li et al., 2014)
Most notably, prophylactic intraperitoneal administration of GR1SN was able to delay the onset of PTB induced by intrauterine LPS in mice, and was associated with altered cytokine and chemokine expression in various gestational tissues, including myometrium. (Yang et al., 2014)

A clinical trial of women receiving oral probiotic supplements (VSL#3), comprised of Lactobacilli, Bifidobacterium, and Streptococcus strains during late gestation demonstrated an alteration in the bacterial components detected in vaginal swabs, demonstrating a more stable microbiota as compared to control patients. IL-4 and IL-10 expression in vaginal samples of women who took VSL#3 were maintained while a decrease in levels of these cytokines were observed in control samples (Vitali et al., 2012). In another in vitro experiment, combination treatment of L. rhamnosus GR-1 and L. reuteri RC-14 exhibited strong antifungal effects against vulvovaginal candidiasis-causing Candida glabrata isolates, (Chew et al., 2015) suggesting a protective function of vaginal probiotics to microbial infections.

Overall, it is becoming increasingly more evident that the commensal vaginal bacteria hold many protective functions in immune regulation by inducing anti-inflammatory responses from various human tissues, including blood leukocytes, gut epithelia, cervical, placental, and uterine cells. This immune regulation in gestational tissues is an important target for therapeutic interventions of infectious-induced sPTL as it is well-established that labouring processes are highly regulated by inflammatory molecules like cytokines that activate labour-associated molecules. Although exact mechanisms by which these effects are exerted have yet to be determined, there is increasing evidence that active compounds within lactobacilli secretions play a crucial role. Prophylactic use of lactobacilli species and their secreted components may offer new therapeutic solutions in targeting the immune regulation of infectious PTL.
1.8 Rationale and Hypothesis

1.8.1 Rationale

Previous *in vivo* experiments have demonstrated that prophylactic administration of GR1SN prior to intrauterine infusion of LPS delayed the onset of PTB in mice and lowered the expression of inflammatory cytokines in various gestational tissues. Similarly, *in vitro* experiments showed the inhibitory effect of GR1SN on cytokine secretion by primary human decidual, placental cells and leukocytes. The inflammatory response was greatest in the myometrium following LPS administration, which suggested a crucial role in myometrial immune regulations for the onset of LPS-induced PTB. The active components within GR1SN have not been identified, and the mechanisms by which they exhibit cytokine suppression in host cells are unknown. Through this thesis, I sought to examine the effects of prophylactic treatment of human myometrial cells by GR1SN for suppression of LPS-induced cytokine secretions. Examination of such pathways and identification of the active components of GR1SN will offer clearer understanding of potential molecular targets in the myometrium for preventing PTB in human.

1.8.2 Hypothesis & Objectives

I hypothesize that GR1SN contains active components that can be used *in vitro* to suppress LPS-induced cytokine secretions by myometrial cells.

The two main aims of this study were to (1) examine the *in vitro* effects of GR1SN on LPS-induced cytokine secretion by human myometrial cells and (2) investigate the mechanisms behind the active components of GR1SN in myometrial cells. Additionally, a third aim was added to (3) test whether prophylactic administration of GR1SN can prevent the onset of PTB induced through systemic administration of LPS in pregnant CD-1 mice.
CHAPTER 2: MATERIALS AND METHODS

A. IN VITRO EXPERIMENTS

2.1 MRS agar and broth preparation
De Man, Rogosa, and Sharpe (MRS) broth was prepared by mixing 55g of MRS powder (BD, Ontario, Canada) in 1L of double deionized water. For MRS agar plates, 15g of agar powder was added to the broth concoction. Broth and agar were autoclaved for sterility.

2.2 Lactobacilli supernatant (SN) preparation and modification

2.2.1 MRS-based SN (GR1SN-MRS)
Lactobacillus rhamnosus GR-1 was grown anaerobically at 37°C for 48h on MRS agar plates and streaked for purity. Single colonies were transferred to 10mL of sterile MRS broth and anaerobically incubated for 12.5h at 37°C to mid-exponential phase at OD$_{620nm}$ ~0.9 (representing ~$10^8$-$10^9$ cfu/mL of bacteria) and centrifuged at 4000g for 10min at 4°C. The supernatant (GR1SN) was passed twice through 0.22µm pore filters for removal of residual bacteria.

2.2.2 PBS-based GR1SN (GR1SN-PBS)
At the time of GR1SN-MRS collection, the bacteria pellet was washed three times in PBS without calcium and magnesium and incubated in PBS with calcium and magnesium for 2h at 37°C under anaerobic conditions. SN was collected by centrifugation at 4000g for 10min and filtered twice through a 0.22µm filter to remove residual bacteria.

2.2.3 GR1SN Size Fractionation
Serial size filtration was conducted using Amicon Ultra membrane filtration tubes (EMD Millipore, Ontario, Canada). 50kDa tubes were filled with GR1SN and centrifuged at 4000g for 10 min, following manufacturer recommendations. Filtrate (< membrane pore size) was collected
and was filtered using 10kDa tubes at 4000g for 40 min, and filtrate from 10kDa tubes was
further fractioned through 3kDa tubes at 4000g for 10 min, following manufacturer
recommendations. Individual residues (> membrane pore size) were collected and resuspended to
starting volume using original solute.

2.3 Macromolecule Detection & Fractionation in -SN

2.3.1 Protein Detection
Bicinchoninic acid assays (BCA and microBCA, Pierce Biotechnology, Thermo Fisher Scientific,
MA, USA) were used to quantify total protein in GR1SN. Standards were prepared using
manufacturer supplied bovine serum albumin (BSA) for a working range of 20 – 2,000µg/mL for
BCA kits, and 2-40µg/mL for microBCA kits. For BCA kits, 2µL of sample were loaded with
38µL of supplied working reagent into a 96-well plate, which was shaken for 30 seconds using a
plate shaker, and incubated at 37˚C for 30 min. When using microBCA kits, 150µl of sample
were loaded with 150µl of working reagent in a 96-well plate, shaken, and incubated at 37˚C for
2 h. Absorbance readings were conducted using µQuant™ software (BioTek® Instruments, Inc.,
VT, USA) at 562nm following manufacturer recommendations.

2.3.2 Nucleic Acid Detection
Agarose gels were prepared by adding 1.5% (w/v) of agarose powder to 1xTris-acetate-EDTA
(TAE) buffer. Solution was warmed in microwave in three 1 minute cycles to dissolve agarose
powder. 4µl/100mL of RedSafe Nucelic Acid Staining Solution (iNtRON Biotechnology, South
Korea) was added to solution. Solution was left at room temperature for 2-3 minutes to cool, and
poured into mould with appropriate combs for well number and size. Gel was left to polymerize
at room temperature for ~20 minutes. Samples were mixed with 3x DNA loading buffer and
loaded into wells. Gel was run at 100V in buffer chambers filled with 1xTAE. Gels were visualized with a UV imager at 30 second exposure.

2.3.3 Specific elimination of GR1SN macromolecule function

a. **Protein**: Two different approaches were used to remove functional protein from the supernatant. GR1SN was (1) heat-treated for 30 minutes at 95°C to denature all native proteins, or (2) incubated 1:1 (v/v) in trypsin-EDTA for 20 minutes at 37°C for protein degradation: trypsin activity was inhibited by adding DMEM/F-12 media at five times the volume of trypsin.

b. **Nucleic Acid**: To degrade nucleic acids in GR1SN, a synthetic nuclease, Benzonase Nuclease (BN, Sigma-Aldrich, Ontario, Canada), was used. BN targets all forms of DNA and RNA; single-stranded, double-stranded, and circular moieties. BN has no proteolytic activity making it safe for use in solutions with protein. Prior to treatment with BN, GR1SN was diluted 1:1 in a solution of 50mM of Tris-HCl (pH 8.0), 1mM MgCl2, and 0.1mg/mL of BSA (BioShop® Canada, Ontario, Canada) following manufacturer recommendations, and was incubated in various concentrations of BN at 4°C for 16h. BN activity was inhibited using 100mM of guanidine HCl, following manufacturer recommendations.

2.4 In vitro cell culture

2.4.1 Primary human myometrial tissue culture

Myometrial tissue biopsies were collected from term not-in-labour women undergoing elective caesarian section at Mount Sinai Hospital after receiving patient’s consent. (n=5) Primary myometrial cells were prepared by enzymatic digestion and cultured in phenol red-free DMEM/F-12 media (Gibco) supplemented with 10% FBS (Wisent Bio Products, QC, Canada).
At passage 4 confluent myometrial cells were serum starved in DMEM/F-12 + 1% ITS (Gibco). Cells were stimulated with 100ng/mL of LPS (E. coli 055:B5, Sigma) for 24 hours. Cell-conditioned media (CM) were collected and analyzed for secreted cytokine concentrations using a Human 9-plex Cytokine assay (Bio-Rad Laboratories, Ontario, Canada).

2.4.2 hTERT-HM culture
A human myometrial smooth muscle cell line immortalized with human telomerase reverse transcriptase (hTERT-HM, gift from Dr. J. Condon) was seeded into 6-well plates (100,000 cells/well) in phenol red-free DMEM/F-12 media (Gibco) supplemented with 10% FBS (Wisent) and 1% Penicillin/Streptomycin/Amphoterin B (Gibco). When ~ 60% confluent, cells were serum-starved in DMEM/F-12 supplemented with 1% ITS-A (Gibco), and 1% Pen/Strep with Amphoterin B (Wisent). After 8 hours in serum-free media (SFM) to synchronize cell cycles, cells were administered a vehicle or pre-treatment with GR1SN for 16 hours, followed by vehicle or LPS to induce inflammation for 8 hours. CM were collected and analyzed for secreted cytokine concentrations.

2.4.3 TLR-agonist kit (InVivogen)
A human TLR-agonist kit was used to individually stimulate TLR 1-10. (InVivogen, CA, USA) Dose-response experiments were conducted by exposing cells to a range of concentrations of individual agonists for 8 hours following manufacturer’s recommendations. Pam3CSK (TLR1/2 agonist) was tested at 0.01, 0.1, 0.5, and 1.0µg/mL; LPS-EK (TLR4 agonist) was tested at 1, 10, 100, 1000ng/mL; FSL-1 (TLR2/6 agonist) was tested at 1, 10, 100, 1000µg/mL.
2.5 Cytokine Quantification Assays

2.5.1 Enzyme Linked Immunosorbent Assay
Sandwich enzyme-linked immunosorbent assays (ELISA) were used to determine specific cytokine concentrations in CM following various combinations of treatments. Ready-SET-Go!® ELISA kits for human IL-6 and IL-8 were purchased from eBioscience (California, USA). DuoSET® ELISA kits and auxiliary kits for human MCP-1, and G-CSF were purchased from R&D Systems Inc. (MN, USA). Conditioned media were diluted 1:10 for IL-6 and IL-8, and 1:5 for MCP-1 assays using supplied diluent solutions from the manufacturer to ensure the absorbance reading would remain in range of the standard curve. Absorbance readings were conducted using µQuant™ software (BioTek® Instruments) at manufacturer-specified wavelengths.

2.5.2 Human 10-multiplex cytokine assay (Bio-Plex)
Cytokine, chemokine, and growth factor concentrations in CM were determined using a human 10-plex Bio-Plex Multiplex Cytokine Immunoassay (Bio-Rad). The assay measured concentrations of IL-6, IL-8, MCP-1, TNF-α, IFN-γ, IL-2, IL-4, IL-10, G-CSF, and GM-CSF. Data were analyzed using Bio-Plex Manager (version 5.0, Bio-Rad) and results are presented as concentrations (pg/mL).
B. IN VIVO EXPERIMENTS

2.6 Animals
All animal experiments were approved by the Animal Care Committee of Toronto Centre for Phenogenomics (Animal Use Protocol 0164H). Guidelines set by the Canadian Council for Animal Care were strictly followed in handling mice. Animals were housed in a pathogen-free, humidity controlled 12h light, 12h dark cycle facility with free access to food and water. Female CD-1 mice were naturally bred; the morning of vaginal plug detection was designated as gestational day (GD) 1 of full term 19-20 days.

2.7 LPS preparation
Stock LPS (E. coli 055:B5, Sigma) was received at 1mg/mL, which was diluted to in PBS without calcium or magnesium to working concentration.

2.8 Intraperitoneal injections
Each mouse received 1.5mg/kg of LPS in saline per intraperitoneal injection, which resulted in low maternal morbidity, while maintaining effectiveness in inducing preterm delivery. Intraperitoneal GR1SN injections (200µl/30g\textit{mouse}) were conducted on GD14, followed by injections of LPS (1.5mg/kg\textit{mouse}) 24h later, on GD15 and 30minutes – second injection of GR1SN. Mice were anesthetized through inhaled isoflurane and solutions were injected into the left upper quadrant of the abdominal cavity. Care was taken to avoid puncture of pups as well as the femoral artery.

2.9 Animal Groups
(1) Vehicle control: Intraperitoneal injections of MRS broth and saline were administered as vehicle for GR1SN and LPS respectively. This group assesses whether there are components within the MRS broth or volume-dependent effects that alter the results.
(2) GR1SN control: Intraperitoneal injections of GR1SN on GD14 and 15 were administered in conjunction with saline (LPS vehicle) on GD15. This group tests for potential effects of GR1SN to ensure it does not induce PTL.

(3) LPS control: This group received intraperitoneal injections of LPS on GD15 without prior GR1SN or MRS broth. This was done to confirm the occurrence of PTL as a result of systemic inflammation and to establish grounds for comparison of the treated group in its benefitting effects of PTL prevention.

(4) GR1SN + LPS treatment: This treatment group received two intraperitoneal injections of GR1SN on GD14 and 15 followed by LPS injections on GD15. All groups were observed at 2hour intervals for the first 24hours, at 4hour intervals until 48hours, and at 12hour intervals until 72hours. Animals that did not deliver preterm were sacrificed at term (GD18.75).

C. STATISTICAL ANALYSIS
All statistical analysis performed in this study were carried out using PRISM software (version 5). Cytokine concentrations were analyzed with One-way ANOVA followed by Dunnett post-test comparing to negative or positive controls accordingly. Data are expressed as mean values ± SEM. P<0.05 was considered statistically significant.
CHAPTER 3: RESULTS

A. IN VITRO EXPERIMENTS

3.1 Establishing an appropriate cell line & inflammatory stimulus

To determine myometrial immune response, CM from primary human myometrial cells stimulated with LPS (100ng/mL) was analyzed using a human BioPlex assay for 9 cytokine markers of inflammation. Cytokines IL-6, IL-8, and MCP-1 were significantly induced in response to LPS stimulus (Figure 1a). However, due to large patient-to-patient sample variation, (Figure 1b) we decided to employ a human myometrial smooth muscle cell line immortalized with telomerase reverse transcriptase (hTERT-HM) for future experiments. LPS treatment (100ng/mL) of hTERT-HM cells for 8 hours resulted in significantly induced IL-6, IL-8, and MCP-1 secretions, (Figure 2) comparable to results of cytokine induction by LPS in primary myometrial cells, human trophoblasts (Yeganegi et al., 2010), human decidual cells (Li et al., 2014), and maternal peripheral blood leukocytes. (Meshkibaf et al., 2015) Such similarities validated the use of hTERT-HM for all future in vitro experiments.

3.2 GR1SN-MRS pre-treatment attenuates LPS-induced inflammatory responses in hTERT-HM cells

Previous in vivo experiments demonstrated that injections of GR1SN-MRS alone did not alter gestational length, maternal morbidity, or fetal weight but that prophylactic administration of GR1SN-MRS prior to local LPS challenge delayed the onset of LPS-induced PTL by 43%. (Yang et al., 2014) Delayed PTL was found to be associated with significantly reduced expression of IL-6, TNF-α, CSF-2, IL-3, IL-9, IL-12, IL-13, and IL-17 in the myometrium of treated mice. To examine potential anti-inflammatory effects of GR1SN-MRS on human
myometrium, hTERT-HM cells were pre-treated \textit{in vitro} with 1, 2, or 5\% v/v GR1SN-MRS, followed by induction of inflammatory response by LPS (100ng/mL),

CM was collected and IL-6, IL-8, or MCP-1 cytokine concentrations were analyzed by ELISAs. Three important observations were made. First, GR1SN-MRS administration alone for 24 hours significantly induced IL-6 secretion in a dose-dependent manner, resulting in 6.2-fold increase at 5\% v/v compared to hTERT-HM cells treated with SFM (negative control). IL-8 and MCP-1 secretions were not affected by GR1SN-MRS exposure. Secondly, concurrent administration of GR1SN-MRS with LPS did not result in suppression of IL-6, IL-8, or MCP-1 secretions relative to LPS (100ng/mL). Thirdly, myometrial cells exposed to low dose of GR1SN-MRS (1\% v/v) for 16 hours prior to LPS administration, secreted significantly lower levels of IL-8 (54\%, P<0.001) and MCP-1 (65.5\%, P<0.001) as compared to LPS treatment. (Figure 3) IL-6 secretions were 36\% lower following 1\% v/v GR1SN-MRS pre-treatment, but this was not statistically significant by one-way ANOVA analysis with Dunnett’s Multiple Comparison post-test.

\subsection*{3.2.1 Identification of macromolecular compounds in GR1SN-MRS}

The macromolecular components of GR1SN-MRS that potentially influenced the secretion of inflammatory cytokines by myometrial hTERT-HM cells were examined. BCA assays, which quantify total protein in solution, detected 2000\(\mu\)g/mL of protein in MRS broth, and 4000\(\mu\)g/mL of protein in GR1SN-MRS (Figure 4a) and the presence of nucleic acids were visualized with a 1.5\% agarose gel (Figure 4b). To examine individual contributions of each macromolecular component, GR1SN-MRS was differentially modified before hTERT-HM pre-treatment. hTERT-HM cells pre-treated with heat-incubated GR1SN to degrade proteins (‘Heat’, Figure 5) showed cytokine secretion values similar to non-heat deactivated crude GR1SN-MRS. (‘1\%’, Figure 5)
Figure 1. Cytokine secretion by primary human myometrial cells in response to LPS stimulus. Conditioned media from untreated (control) cells and cells treated with 100ng/mL of LPS for 24 hours were analyzed for cytokine concentrations using a Luminex human 9-plex cytokine assay. Secreted cytokine concentrations are shown as absolute concentrations in pg/ml. Individual dots represent different patients (n=5). Statistical significance was determined by individual t-tests (*P<0.05; **P<0.01). This experiment was performed with assistance from Dr. O. Shynlova and A. Dorogin.
**Figure 2. Comparative dose and time responses following LPS administration in hTERT-HM cells.**

**a)** Cells were treated with various doses of LPS (0.1-1000 ng/mL, grey to black bars) for 8 hours. Dose response values are shown as fold change (mean ± SEM) relative to untreated vehicle (white bars). Significance was determined by One-way ANOVA followed by Dunnett post-test compared to vehicle control.

**b)** Cells were treated with 100 ng/mL of LPS for various time points. Data are shown as absolute concentrations [pg/mL]. Significance was determined by t-tests between vehicle and LPS values at individual time points. (*P<0.05, **P<0.01, ***P<0.001)
Figure 3. Effect of GR1SN-MRS treatment on hTERT-HM cell cytokine secretion following LPS stimulus. hTERT-HM cells were treated with various doses of GR1SN-MRS (1, 2, 5% v/v, shades of orange) or MRS broth (1% v/v, veh) for 16 hours prior to or concurrently with (1%, v/v, Co-1%, brown bars) LPS (100ng/mL). Cytokine secretions are shown as fold change (mean ± SEM) relative to untreated vehicle (white bars). Statistical significant difference was determined by One-way ANOVA followed by Dunnett’s post-test relative to LPS stimulus (black bars). (*P<0.05; **P<0.01; *** P<0.001) (n=9)
However, GR1SN-MRS modified with trypsin lost its ability to prevent cytokine induction in hTERT-HM cells following LPS stimulus, (‘Trypsin’, Figure 5) as characterized by high concentrations of secreted IL-6, IL-8, and MPC-1 comparable to levels secreted by hTERT-HM cells treated with LPS only. (‘LPS+’, Figure 5)

Degradation of nucleic acid in GR1SN-MRS following incubation with BN at 50U/mL and 100U/mL was verified using a 1.5% agarose gel. (Figure 4d) Pre-treatment with BN-modified GR1SN-MRS demonstrated similar suppression of LPS-induced cytokine secretions by hTERT-HM cells as compared to non-BN-treated crude GR1SN-MRS, (‘BN’, “1%”, Figure 5) which suggests a lack of immune regulatory effect exerted by nucleic acids in GR1SN-MRS.

3.3 MRS broth influences myometrial inflammation
Data presented above indicated the potential role of proteins in GR1SN-MRS on the induction of IL-6 secretions by hTERT-HM cells. To test the effect of MRS broth alone, various doses of broth ranging from 0.1, 0.5, 1, 5% v/v were administered to hTERT-HM cells. It was determined by cytokine analysis that similar to the effects of GR1SN-MRS, IL-6 concentrations were significantly (P<0.05) induced by MRS alone as compared to the negative control (SFM), resulting in a 4.5-fold induction following 5% v/v MRS treatment. (Figure 6) A significant reduction of IL-6 secretion (57.6%, P<0.05) was also observed following 0.1% v/v MRS pre-treatment prior to LPS stimulus (100ng/mL). Secretion of chemokines IL-8 and MCP-1 were not induced by MRS broth alone, but MRS pre-treatment at 1%, 2%, 5% v/v resulted in significant suppression of both cytokines (P<0.001) when administered prior to LPS stimulus. (Figure 6)
Figure 4. Characterizing macromolecules in GR1SN-MRS

a) Protein concentrations in MRS broth (1941.7 µg/mL) and GR1SN-MRS (3946.7 µg/mL) were detected using a BCA assay. (n=3)

b) 1.5% agarose gel depicting nucleic acid content in GR1SN-MRS. A band of nucleic acid is visible in unmodified GR1SN (white band, SN) but is absent in GR1SN treated with BN (SN+BN). L, Ladder.
Figure 5. Relative cytokine secretions by hTERT-HM cells pre-treated with modified GR1SN-MRS prior to LPS stimulus. hTERT-HM cells were pre-treated with non-modified GR1SN-MRS (orange bars), or with GR1SN-MRS modified to degrade nucleic acids (BN, green bars), denature protein structures (Heat, pink bars) or degrade proteins (Trypsin, violet bars) prior to LPS (100ng/mL). Cytokine secretions are shown as fold change (mean ± SEM) relative to untreated cells (white bars). Statistical significance was determined by One-way ANOVA followed by Dunnett’s post-test relative to LPS stimulus (black bars). (*P<0.05; **P<0.01; ***P<0.001)
3.4 GR1SN-PBS pre-treatment can suppress LPS-induced cytokine and chemokine secretions by hTERT-HM cells

Following observation that MRS broth alone exerted immune regulatory effects on hTERT-HM cells, PBS-based GR1SN was developed as described in Methods. Next, GR1SN-PBS was administered to hTERT-HM cells before LPS insult (100ng/mL), CM was collected and analyzed for cytokine secretions by ELISA. GR1SN-PBS treatment alone did not result in increased secretion of IL-6, IL-8, or MCP-1 as compared to SFM control. Concurrent treatment of cells with both GR1SN-PBS and LPS (100ng/ml) did not inhibit cytokine secretions by myometrial cells in response to LPS stimulus. GR1SN-PBS pre-treatment for 18 hours prior to LPS showed significant (p<0.05) suppression of IL-6 by 47.11%, IL-8 by 34.8% and of MCP-1 by 38.4% as compared to LPS induction. (Figure 7)

3.4.1 Identification of functionally active macromolecules in GR1SN-PBS

BCA analysis of GR1SN-PBS found 22µg/mL of protein in the supernatant, a significant reduction of total protein content relative to GR1SN-MRS (Figure 8a). GR1SN-PBS was heat-treated or incubated with trypsin to remove functional protein activity. hTERT-HM pre-treated with either of heat-treated or trypsin-incubated GR1SN-PBS did not demonstrate suppressed cytokine secretions following LPS stimulus indicating a functional capacity of GR1SN-PBS proteins on suppressing LPS-induced cytokine secretions by hTERT-HM cells (Figure 9).
Figure 6. Effect of MRS broth on cytokine secretion in hTERT-HM. hTERT-HM cells were treated with various doses of MRS broth (0.1-5% v/v, shades of yellow) with or without LPS (100ng/mL). Cytokine secretions are shown as fold change (mean ± SEM) relative to untreated vehicle (white bars). Statistical significance was determined by One-way ANOVA followed by Dunnett’s post-test relative to LPS stimulus (black bars). (*P<0.05; **P<0.01; ***P<0.001) (n=4)
Figure 7. Effect of GR1SN-PBS treatment on cytokine suppression following LPS stimulus. hTERT-HM cells were treated with various doses of GR1SN-PBS (1, 2, 5% v/v, shades of blue) 16 hours prior to or concurrently with (Co-SN, dark blue bars) LPS (100 ng/mL). Cytokine secretions are shown as fold change (mean ± SEM) relative to untreated vehicle (white bars). Statistical significant difference was determined by One-way ANOVA followed by Dunnett’s post-test relative to LPS stimulus (black bars). (*P<0.05; **P<0.01; ***P<0.001) (n=9)
3.4.2 Size Fractionation of GR1SN-PBS

To isolate the active component of GR1SN-PBS, the supernatant was centrifuged using filtration tubes at 3 different membrane sizes; 3kDa, 10kDa, 50kDa. The amount of protein within each fraction was quantified by BCA analysis. From the initial 22µg/mL of total protein, 4.24µg/mL was in the >50kDa fraction, and 13µg/mL was found in the <50kDa fraction. The filtrate of 10kDa (<10kDa) contained 12µg/mL of protein, and 13.5µg/mL of protein was <3kDa. Pre-treatment of hTERT-HM cells with the >50kDa fraction resulted in significant suppression of MCP-1 by 50.4% (P<0.001). Pre-treatment with the <50kDa fraction was effective in significantly suppressing IL-6 and IL-8 by 52.7% (P<0.05), MCP-1 by 54.6% (P<0.001). Pre-treatment with the <10kDa fraction did not result in significant IL-6 suppression, though IL-8 secretions were reduced by 50.5% (P<0.01) and MCP-1 secretion was suppressed by 64.8% (P<0.001). Fractions smaller than 3kDa also retained activity to significantly (P<0.01) suppress cytokines and chemokines, with IL-6 secretions reduced by 32.3%, IL-8 by 53.5%, and MCP-1 by 50%. Alternatively, serial fractionation steps were also performed where all supernatant were initially passed through the 50kDa filter, and the filtrate (<50kDa) was then passed through a 10kDa filter, and again through a 3kDa filter. This resulted in protein fractions sized between 10 and 50kDa (3.73µg/mL) and between 3 and 10kDa (6.17µg/mL). Pre-treatment of hTERT-HM cells with each of the fractions prior to LPS stimulus resulted in significant suppression of IL-6 secretion by the proteins from two fractions sized 3<x<10kDa and >50kDa. IL-8 was significantly suppressed by hTERT-HM pre-treated with two fractions: <3kDa and 3<x<10kDa. MCP-1 was significantly suppressed following hTERT-HM pre-treatment with 10<x<50kDa and >50kDa fractions. (Figure 10)
Figure 8. Quantifying proteins in GR1SN-PBS a) Protein concentrations in GR1SN-MRS (3946.7 µg/mL) and GR1SN-PBS (22.6 µg/mL) were detected using a BCA assay. (n=3)
Figure 9. Effect of modified GR1SN-PBS on LPS-induced cytokine secretions. hTERT-HM cells were treated with non-modified GR1SN-PBS (blue bars), or with GR1SN-PBS modified to denature protein structures (Heat, green bars) or degrade proteins (Trypsin, violet bars), with or without LPS stimulus (100ng/mL). Cytokine secretions are shown as fold change (mean ± SEM) relative to untreated cells (white bars). Statistical significance was determined by One-way ANOVA followed by Dunnett’s post-test relative to LPS control (black bars). (*P<0.05; **P<0.01; ***P<0.001) (n=4)
Figure 10. Effect of hTERT-HM pre-treatment with various GR1SN-PBS size fractions on LPS-induced cytokine secretions. hTERT-HM cells were pre-treated with non-modified GR1SN-PBS (blue bars), or with size-fractioned GR1SN-PBS prior to LPS stimulus (100ng/mL). GR1SN-PBS was separated into fractions between 3 and 10kDa ([3,10kDa], blue horizontal stripes), between 10 and 50kDa ([10,50kDa], blue vertical stripes), or greater than 50kDa (>50kDa, blue diagonal stripes). Cytokine secretions are shown as fold change (mean ± SEM) relative to untreated cells (white bars). Statistical significance was determined by One-way ANOVA followed by Dunnett’s post-test relative to LPS control (black bars). (*P<0.05; **P<0.01; ***P<0.001) (n=3)
3.5 Low-dose TLR agonist pre-treatment attenuates LPS-induced inflammatory responses in hTERT-HM cells.

As described above, concurrent incubation of neither GR1SN-MRS nor GR1SN-PBS with LPS led to suppression of LPS-induced cytokine secretion by hTERT-HM, while GR1SN pre-treatment of GR1SN-PBS for 16 hours inhibited LPS-induced inflammatory responses. (Figure 3, Figure 7) Such findings led me to hypothesize that the active components within GR1SN do not function by direct effect to inhibit LPS stimulus, but rather offer immune protection through cell-mediated pathways to suppress LPS-induced cytokine secretion.

Previous studies have demonstrated that GR1SN-MRS induced secretions of anti-inflammatory cytokines such as IL-4, IL-10 (Yeganegi et al., 2010; Yang et al., 2014) or growth factor G-CSF, (Kim et al., 2006; Yeganegi et al., 2011; Meshkibaf et al., 2015) to regulate the overall ratio of pro- to anti-inflammatory state in treated cells. However, it was found using a 10-plex human cytokine multiplex assay (BioPlex), that hTERT-HM cells do not secrete detectable levels of anti-inflammatory cytokines or growth factors following GR1SN treatment (Table 1).

Alternatively, mechanisms of endotoxin tolerance also result in cytokine suppression through cell mediated pathways without influencing secretion of anti-inflammatory cytokines. Tolerance results through repeated stimulus from same or similar agonists. It has been previously reported that TLR4, a known receptor for LPS, can become desensitized to repeated LPS stimulus (homo-desensitization) (Lehner et al., 2001; Dobrovolskaia et al., 2003a) or to repeated stimulus of other TLRs that share similar downstream inflammatory pathways, such as TLR2 (hetero-desensitization). (Lehner et al., 2001; Dobrovolskaia et al., 2003a) Thus, I hypothesized that GR1SN-mediated activation of TLRs in hTERT-HM cells during the pre-treatment period may elicit tolerance to a subsequent TLR4 stimulation by LPS.
Table 1. 10-plex BioPlex cytokine assay of media conditioned by hTERT-HM cells treated with GR1SN-MRS. Secreted cytokine concentrations in CM were detected from cells untreated (basal levels), exposed to GR1SN (GR1SN-induced), or LPS-induced (LPS-induced). Average concentrations from 5 experiments and their range are shown in pg/mL. OOR<, values below lowest detectable concentration set by standards.

<table>
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<tr>
<th>Cytokine</th>
<th>Lowest detectable concentration [pg/mL]</th>
<th>Basal levels Average (min-max) [pg/ml]</th>
<th>GR1SN-induced Average (min-max) [pg/ml]</th>
<th>LPS-induced Average (min-max) [pg/ml]</th>
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<tr>
<td>IL-6</td>
<td>1.41</td>
<td>159.2 (91.3-327.4)</td>
<td>135.1 (95.5-252.5)</td>
<td>827 (424-1293)</td>
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<tr>
<td>IL-8</td>
<td>1.41</td>
<td>565.4 (150.1-1867.1)</td>
<td>1177.7 (127.1-4807.3)</td>
<td>2044 (1152-3330)</td>
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<tr>
<td>MCP-1</td>
<td>3.36</td>
<td>199.1 (136.9-282.7)</td>
<td>169.9 (135.7-219.8)</td>
<td>664 (548-911)</td>
</tr>
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<td>IFN-γ</td>
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<td>OOR&lt;</td>
<td>OOR&lt;</td>
<td>OOR&lt;</td>
</tr>
<tr>
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</tr>
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3.5.1 Low-dose LPS and Pam3CSK pre-treatment can suppress LPS-induced cytokine secretion

To test this hypothesis, I first established if hTERT-HM cells could gain tolerance to repeated stimuli of the same agonist (homo-desensitization). Low-dose LPS (0.01, 0.1, 1.0ng/mL) was used to pre-treat hTERT-HM cells for 16 hours followed by a single high dose of LPS (100ng/mL) to induce inflammatory signalling. Conditioned media from treated cells were analyzed by ELISA for inflammatory cytokines IL-6, IL-8, and MCP-1. I found that hTERT-HM pre-treatment with 0.1ng/mL of LPS prior to 100ng/mL of LPS suppressed secretions of IL-6 by 43.5% (P<0.05), IL-8 by 38.9% (P<0.05), and MCP-1 by 31% (P<0.01) (Figure 11). Confirmation that TLR4-induced inflammatory responses to LPS stimulus is reduced in myometrial cells led to further experiments to determine if other TLR agonists were capable of suppressing LPS-induced cytokine secretion. Manufacturer-recommended doses of functional Pam3CSK (TLR1/2 agonist) at 0.01, 0.1, 0.5, and 1.0µg/mL and for FSL-1 (TLR2/6 agonist) at 1.0, 10, or 100µg/mL were used to treat hTERT-HM cells for 8 hours. I found that Pam3CSK-induced secretion of cytokines in a dose dependent manner; 0.5µg/mL of Pam3CSK induced IL-6 secretions by 3.7-fold (P<0.001), IL-8 by 2.9-fold (P<0.01), and MCP-1 by 2.7-fold (P<0.01), (Figure 12) whereas FSL-1 was not able to induce any inflammatory cytokine secretions by hTERT-HM cells. (Figure 13) Using low dose Pam3CSK (0.01µg/mL) which did not induce IL-6, IL-8, and MCP-1 secretions as compared to SFM control, hTERT-HM cells were pre-treated for 16 hours prior to a single high dose LPS (100ng/mL). ELISA analysis of CM demonstrated suppression of LPS-induced pro-inflammatory cytokines IL-6 by 38% (P<0.01), IL-8 by 41% (P<0.05), and MCP-1 by 43% (P<0.05) as compared to LPS stimulus. (Figure 14)
Figure 11. Effect of low-dose LPS pre-treatment prior to high-dose LPS stimulus on cytokine secretions by hTERT-HM cells. Cells were pre-treated with 0.1µg/mL of LPS with (checkered) or without a subsequent LPS stimulus (100ng/mL). Secreted cytokine levels are shown as fold change (mean ± SEM) relative to untreated vehicle (white bars). Statistical significance was determined by One-way ANOVA followed by Dunnett’s post-test relative to LPS stimulus (black bars). *P<0.05; **P<0.01; ***P<0.001. (n=5)
Figure 12. Effect of TLR1/2 agonist (Pam3CSK) dose on cytokine secretion by hTERT-HM cells. Cells were treated with various doses of Pam3CSK (P3K, 0.01-1 µg/mL, violet bars) for 8 hours. Secreted cytokine levels are shown as fold change (mean ± SEM) relative to untreated vehicles (white bars). Statistical significance was determined by One-way ANOVA followed by Dunnett’s post-test. **P<0.01; ***P<0.001. (n=5)
Figure 13. Effect of TLR2/6 agonist (FSL-1) dose on cytokine secretion by hTERT-HM cells. Cells were treated with various doses of FSL-1 (1-100 µg/mL, pink bars) for 8 hours. Secreted cytokine levels are shown as fold change (mean ± SEM) relative to untreated vehicle (white bars). Statistical significance was determined by One-way ANOVA followed by Dunnett’s post-test. **P<0.01; ***P<0.001. (n=5)
Figure 14. Effect of low-dose Pam3CSK pre-treatment prior to high-dose LPS stimulus on cytokine secretions by hTERT-HM cells. Cells were pre-treated with 0.01\(\mu\)g/mL of Pam3CSK (P3K) with (checkered) or without LPS stimulus (100ng/mL). Secreted cytokine levels are shown as fold change (mean ± SEM) relative to untreated vehicle (white bars). Statistical significance was determined by One-way ANOVA followed by Dunnett’s post-test relative to LPS stimulus (black bars). *P<0.05; **P<0.01; ***P<0.001. (n=5)
3.6 Cytokine regulation in myometrial cells by GR1SN is unique as compared to four other non-L. rhamnosus GR-1 lactobacilli species.

Since TLR2 is not exclusive to L. rhamnosus GR-1 but is common to bacterial lipoproteins such as LTA, I examined if non-GR-1 lactobacilli strains would also demonstrate similar inhibitory effects on cytokine secretion. For this experiment, I used 4 other strains of probiotic lactobacilli, L. rhamnosus GG, L. lactis, L. casei, and L. reuteri RC-14 provided by Dr. Sung Kim (Western University, London, Ontario).

All strains were cultured in MRS broth (as described above for GR-1) and their PBS-based supernatants were used to pre-treat hTERT-HM cells in the same manner as was described for GR1SN. ELISA analysis of CM from hTERT-HM cells pre-treated with different lactobacilli-SNs demonstrated that SN from L. rhamnosus GG, lactis, casei, and reuteri RC-14 were not able to suppress LPS-induced cytokine secretions, and that GR1SN-PBS is a unique suppressor of LPS-induced cytokine secretion by hTERT-HM cells (Figure 15).
Figure 15. Effect of five different lactobacilli strains on cytokine suppression in hTERT-HM cells stimulated with LPS. Cells were pre-treated with various doses of distinct Lactobacilli species (blue bars) with (checkered) or without LPS stimulus (100ng/mL). Results are demonstrated as fold change (mean ± SEM) compared to untreated vehicle control (white bars). Statistical significance was determined by One-way ANOVA followed by Dunnett’s post-test compared to LPS stimulus (black bars). *P<0.05; **P<0.01; ***P<0.001. GG, L rhamnosus GG; Lactis, L. lactis; Casei, L. casei; RC-14, L. reuteri RC-14. (n=9)
B. IN VIVO EXPERIMENTS

3.7 In vivo effects of GR1SN-MRS pre-treatment to delay the onset of LPS-induced PTL

Previous studies have reported the successful role of prophylactic administration of systemic GR1SN-MRS to delay the onset of local LPS-induced PTB in CD-1 mice. In the current study, I examined whether the administration of systemic GR1SN-MRS can also delay PTL induced by systemic low dose infection. Timed pregnant animals were divided into 4 groups: 1) MRS broth 2) GR1SN-MRS control 3) LPS and 4) GR1SN-MRS + LPS. Deliveries made during the first 24h post-LPS injection were classified as preterm.

Group 1: MRS broth had no effect on inducing PTL. Intraperitoneal injection of MRS broth (200µL) on GD14 and again on GD15 immediately followed by saline injections (100µL) showed no indication of preterm labour in treated mice (n=5). All mice were healthy and active until they were sacrificed at GD18.75. No fetuses were resorbed.

Group 2: GR1SN-MRS does not cause maternal mortality nor induce PTL. Intraperitoneal injection of GR1SN-MRS (200µL) on GD14 and again on GD15 immediately followed by saline injections (100µL) showed no indication of preterm labour in treated mice (n=5). All mice were healthy and active until sacrificed at GD18.75. No fetuses were resorbed.

Group 3: Systemic LPS injections will induce PTL. Intraperitoneal injections of LPS (1.5mg/kg, 100µl) on GD15 resulted in 70% of treated mice (n=10) delivering preterm. Despite the definition of preterm birth as delivery within 48 hours of LPS injection, it was observed that all 7 mice that delivered preterm did so within 24 hours of LPS administration.

Group 4: GR-1SN-MRS + LPS Treatment. Intraperitoneal injections of GR1SN on GD14 and again on GD15 immediately followed by LPS injections resulted in PTB in 7 of 12 treated mice (58%). All mice appeared healthy, active and nesting. There were 6 mice that underwent partial
delivery, where only a portion of the uterine horn was emptied. These were classified as having delivered preterm, and are included in the 58%. All mice that responded and delivered at term did so at GD18.75. The LPS group was compared to the prophylactic GR1SN treatment group using Fisher’s exact test. It was determined that the change in rate of PTB due to prophylactic GR1SN was not significant (p=0.67) as compared to LPS control. (Table 2)

Table 2. The effect of prophylactic GR1SN-MRS on delivery rates of LPS-induced preterm birth in pregnant CD-1 mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Preterm Delivery Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>GR1SN-MRS</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>LPS (1.5mg/kg)</td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td>GR1SN-MRS + LPS</td>
<td>12</td>
<td>58</td>
</tr>
</tbody>
</table>

3.8 In vivo effects of GR1SN-PBS pre-treatment to inhibit LPS-induced PTL

Similarly, mice received intraperitoneal injections of GR1SN-PBS prior to intraperitoneal injections of LPS to determine if GR1SN-PBS can delay PTB induced by systemic LPS administration. Timed pregnant mice were divided into 4 groups: 1) saline control, 2) GR1SN-PBS, 3) LPS (1.5mg/kg), and 4) GR1SN-PBS + LPS (1.5mg/kg). Deliveries made during the first 24h post-LPS injection were classified as preterm.

Group 1: Saline had no effect on inducing PTL. Intraperitoneal injection of PBS (300µL) on GD14 and again on GD15 immediately followed by saline injections (200µL) showed no indication of PTL in treated mice (n=4). All mice were healthy and active until they were sacrificed at GD18.5.

Group 2: PBS-based GR-1SN did not cause maternal mortality nor induce PTL. Intraperitoneal injection of GR-1SN (300µL) on GD14 and again on GD15 immediately followed by saline
injections (200µL) showed no indication of preterm labour in treated mice (n=7). All mice were healthy and active until sacrificed at GD18.5. No fetuses were resorbed.

(3) Systemic LPS injections induced PTL: Intraperitoneal injections of LPS (1.5mg/kg, 200µl) on GD15 resulted in 100% of treated mice (n=10) to go into deliver preterm within 24 hours of LPS administration, indicating “treatment-to-delivery interval” of 22 hours. (Table 3)

(4) GR-1SN + LPS Treatment: Pregnant mice (n=10) received intraperitoneal injections of GR1SN (300µL) on GD14 and again on GD15 immediately followed by LPS injections (2 mg/kg in 200µl) which resulted in 80% PTB. We did not observe any significant effect of prophylactic GR1SN-PBS treatment in preventing LPS-induced PTB in pregnant CD-1 mice by performing a Fisher’s exact test to compare the LPS group with the GR1SN treatment group (p=0.47).

Table 3. The effect of prophylactic GR1SN-PBS on delivery rates of LPS-induced preterm birth in pregnant CD-1 mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Preterm Delivery Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>GR1SN-PBS</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>LPS (1.5mg/kg)</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>GR1SN-PBS + LPS</td>
<td>10</td>
<td>80</td>
</tr>
</tbody>
</table>
CHAPTER 4: DISCUSSION

4.1 In vitro effects of GR1SN pre-treatment on cytokine suppression in hTERT-HM cells stimulated with LPS.

Through my in vitro studies, I have (1) demonstrated the retained suppressive effect of the secreted compounds of *Lactobacillus rhamnosus* GR-1 in a PBS-based solution and introduced endotoxin tolerance as a potential mechanism of action, and (2) observed an effect of MRS broth alone to influence inflammatory signals in hTERT-HM cells.

4.1.1 Endotoxin Tolerance in hTERT-HM cells

Alternatively, previous in vivo experiments also demonstrated that concurrent administration of GR1SN-MRS and LPS did not delay the onset of PTB in pregnant mice. These findings are further supported in the current in vitro study, as concurrent treatment of cells with GR1SN (in either form, MRS or PBS) and LPS does not suppress LPS-induced secretions of cytokines and chemokines. In both in vivo and in vitro settings, preventative effects of GR1SN were only shown when GR1SN was used as a pre-treatment, which suggests the need of a lag period necessary for GR1SN to induce intracellular changes in host cells, altering host responses to subsequent LPS stimulation. Studies of GR1SN in other tissue types, such as monocytes, (Meshkibaf *et al.*, 2015) decidual (Li *et al.*, 2014) and trophoblast cells, (Yeganegi *et al.*, 2009, 2010, 2011) have demonstrated the secretion of anti-inflammatory cytokines such as IL-10 to explain the effects observed by GR1SN pre-treatment. However, neither primary human myometrial cells, nor hTERT-HM cells used in the current study secreted detectable amounts of anti-inflammatory cytokines following GR1SN treatment.

Rather, studies of endotoxin tolerance in human and murine immune cells suggest that reduced expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 is not
necessarily paired with induction of anti-inflammatory cytokines like IL-10. (Sato et al., 2000) “Endotoxin tolerance” refers to a mechanism by which cells become less responsive to a secondary endotoxin (i.e. LPS stimulus) following pre-treatment with low-doses of identical or similar agonists that activate overlapping downstream pathways. Tolerance has been well-established in immune cells by pre-treatments with agonists for TLR2, (Sato et al., 2000) TLR4, (Dobrovolskaia et al., 2003b) and TLR9 (Cowdery et al., 1996; Gao et al., 1999). A recent study elucidated the effects of endotoxin tolerance induced by *Lactobacillus plantarum* treatment in human intestinal cells (Caco-2), characterized by increased expression of regulatory molecules of immune activation such as TOLLIP, SOCS1, SOCS3 and their associated suppression of pro-inflammatory pathways. (Chiu et al., 2013)

I have demonstrated here that GR1SN pre-treatment suppresses LPS-induced cytokine secretions by hTERT-HM cells. As an initial proof-of-principle experiment, I successfully induced endotoxin tolerance in hTERT-HM cells by using sub-stimulatory doses of LPS prior to a high dose simulation with LPS. This resulted in significant suppression of IL-6, IL-8 and MCP-1 as compared to cells treated with a single high dose of LPS. Subsequent experiments to identify the specificity of the pathway were performed as endotoxin tolerance can be induced through TLR2 and TLR4 by bacterial proteins, and through TLR9 by bacterial nucleic acids. Nucleic acids were identified in GR1SN through nucleic acid staining in agarose gels. However, it was found that tolerance to LPS in hTERT-HM cells is not mediated through TLR9 activation by nucleic acids in GR1SN as GR1SN modification with BN incubation to degrade nucleic acid maintained its effect to suppress cytokine and chemokine secretions. Thus, I assume that the active component of SN to be of protein/lipoprotein origin. BCA assays of GR1SN indicated high protein content, which can include lipoprotein moieties secreted or shed by gram-positive
bacteria that activate TLR2 receptors and confer tolerance to subsequent endotoxin stimulus. TLR2 functions as a heterodimer with TLR1 to detect triacylated lipoproteins or with TLR6 to recognize diacylated lipoproteins. In current studies, I employed highly specific synthetic agonists for TLR1/2 (Pam3CSK) and TLR2/6 (FSL-1) to individually pre-treat hTERT-HM cells prior to LPS stimulus. It was found through preliminary dose response experiments that hTERT-HM cells do not respond to FSL-1, suggesting that TLR2/6 activation is not a plausible pathway of endotoxin tolerance in hTERT-HM cells. Conversely, Pam3CSK experiments demonstrated a dose-dependent increase in secretions of pro-inflammatory cytokines by hTERT-HM cells. Pre-treatment of cells with non-stimulatory doses of Pam3CSK was able to significantly suppress LPS-induced secretions of IL-6, IL-8, and MCP-1, indicating a potential role of the TLR1/2 complex in the mediation of cytokine suppression by GR1SN pre-treatment in hTERT-HM cells.

Currently, the exact agonists for TLR2 within GR1SN have not been identified. However, recent studies examining GR1SN exposure to macrophages have identified heat-labile, protein-like components between 30-100kDa to promote G-CSF production through TLR2-dependent mechanisms (Meshkibaf et al., 2015). In the current study, nucleic acid and protein components were identified in GR1SN. Modifications to remove functional protein activity by heat and trypsin incubation effectively ablated GR1SN effect to suppress secretion of LPS-induced pro-inflammatory factors in hTERT-HM cells. Contrastingly, nucleic acid degradation by incubation with BN did not alter GR1SN effect, although the visible band of nucleic acid was removed in the agarose gel. As BN was claimed by the manufacturer to degrade all DNA and RNA, these findings suggested that GR1SN effect can be attributed to protein-like components and not nucleic acids. However, a study examining the effect of ribonucleases, including BN, on RNA stability demonstrated that although BN can reduce the amount of intact mRNA by
approximately 50%, the amount of intact miRNA was unaffected and remained around 100%. (Aryani and Denecke, 2015). This suggested that despite the lack of a visible band of nucleic acid on the agarose gel, there may be unaffected forms of nucleic acid such as miRNA that persist in the GR1SN to affect inflammatory signalling in exposed cells. Furthermore, pre-treatment of hTERT-HM cells with sized fractions of GR1SN-PBS demonstrated uncertain results whereby pre-treatment with fractions greater than 50kDa significantly suppressed IL-6 and MPC-1 secretion to levels comparable to unfractionated (crude) GR1SN-PBS, while IL-8 secretion was not affected.

Lipoprotein sizes vary vastly according to the species from which they are produced and their endogenous functions, which include regulation of host immune response, bacterial virulence, and nutrient acquisition for the bacteria to better survive the host. (Nguyen and Götz, 2016) Lipoproteins are generally produced as pre-pro-lipoproteins, and are matured through lipidation to their functional form. In gram-positive species, they are secreted in extracellular vesicles which then bind to host receptors, or are found anchored on lactobacilli cell wall. (Lebeer et al., 2008) For instance, it was determined that the genome of Lactobacillus johnsonii contains sequences for two lipoproteins, one which may activate CD4+ T-cells and another which holds similar composition to saliva-binding proteins found in S. sanguis. (Pridmore et al., 2004) However, their protein function has not been studied. It is also possible that tissue specificity plays an important role in identifying the functional bacterial lipoprotein.

Alternatively, mechanisms behind endotoxin tolerance can be studied by observing changes in downstream mediators. Endotoxin tolerance has been shown to be established through distinct mechanisms which vary depending on the initial TLR and agonist pair that prime the cells. TLR4-induced endotoxin tolerance operates through mechanisms different from
TLR2-induced tolerance. TLR4-mediated endotoxin tolerance leads to decreased surface expression of the TLR4/MD2 complex, (Nomura et al., 2000; Sato et al., 2000) as well as decreased expression of adaptor proteins MHP-II, and CD86 (Wolk et al., 2000) at the cell membrane, without inducing IL-10 secretions in murine macrophages. Monocyte-line human THP-1 cells tolerized by LPS also show alteration in the NF-kB pathway at downstream sites, reducing activation of ERK1/2 and p38. (Kraatz et al., 1999) However, TLR2-mediated TLR4 desensitization does not result in reduced TLR4/MD2 expression at the cell surface of murine macrophages, but can impair NF-kB dimer formations and JNK activation, thus leading to reduced pro-inflammatory cytokine secretions. (Sato et al., 2000) Moreover, when two different agonists of TLR1/2 (hBD-3 and Pam3CSK), were used to treat human monocytes, it was observed that while both stimuli increased IL-6, IL-8, IL-1β and activated MAPK, increased expression of IL-10 was only observed in cells treated with Pam3CSK and not with hBD-3, further indicating differential cellular responses to agonists. This was associated with a decrease in CD86, and was suggested to be due to selective activation of NF-κB in Pam3CSK-treated cells but not hBD-3. (Funderburg et al., 2011) Endotoxin tolerance can also be induced through GPCR activation in association with G i protein activation, which can suppress LPS-induced NF-κB signaling through increased PI3K and MAPK-phosphatase expression in murine-derived peripheral macrophages. (Fan et al., 2008) As such, the differential influences exerted by distinct priming agonists offer targets which can be explored in future experiments to elucidate the pathways and TLRs that are involved in GR1SN-mediated endotoxin tolerance in hTERT-HM cells.

A potential concern behind endotoxin tolerance may be that suppression of cytokines and chemokines may result in persistent bacterial infection as the normal function of cytokines is to
activate the immune system in response to infection of foreign organisms. In gestation, persistent infection may lead to more detrimental consequences to the fetus than PTB. However, endotoxin tolerance is a complex pathway which involves more than a global suppression of inflammatory cytokines. In vivo experiments of endotoxin tolerance demonstrated that despite reduced cytokine expression, mice were more resistant to gram-negative bacterial infection, (Leon et al., 1992) and a reduced fungal burden following inoculation with Cryptococcus neoformans in tolerized animals, (Rayhane et al., 2000) indicating an active immune system to fight off pathogens. Most of the research on endotoxin tolerance has been performed in immune cells (human and murine monocytes and macrophages) which consistently show attenuated TNF-α expression, but vast inconsistencies persist in the regulation of many other inflammatory markers. (West and Heagy, 2002) These discrepancies have been attributed to LPS purity, type or dose, as well as host cell properties. Indeed, paradoxically, recent studies examining the role of L. rhamnosus GR-1 treatment in bladder cells have shown that probiotic exposure leads to an augmented inflammatory response. (Karlsson et al., 2012)

My work has examined the effects of endotoxin tolerance in human myometrial cells (hTERT-HM), in which TNF-α was produced below detectable levels (lowest detectable concentration: 3.18pg/mL) both at baseline and following LPS stimulus; and GR1SN-PBS pre-treatment resulted in suppressed secretion of all IL-6, IL-8, and MCP-1. The novelty of my work lies in the identification of endotoxin tolerance as a potential mechanism underlying the anti-inflammatory effects observed in hTERT-HM by GR1SN pre-treatment. I have shown that uterine myocytes are sensitive to endotoxin tolerance, a mechanism previously limited to studies in immune and epithelial cells. This further emphasizes the role of the myometrium in maintaining immune homeostasis throughout gestation, and in regulating the onset of labour in
cases preceded by infection. However, it is important to note that probiotic function is highly tissue-specific: I demonstrated that four other lactobacilli species (L. rhamnosus GG, L. lactis, L. casei, and L. reuteri RC-14) did not significantly suppress LPS-induced cytokine secretions in hTERT-HM cells despite their well-established anti-inflammatory effects in other tissue types.

4.1.2 Effect of MRS broth on inflammatory signals in hTERT-HM cells

Interestingly, it was observed that treatment with MRS broth alone can influence inflammatory signals in hTERT-HM cells such suppression of LPS-induced pro-inflammatory factors and an increase in IL-6 secretions, similar to the effects of GR1SN-MRS. MRS broth is a concoction of various factors optimized for the growth of lactobacilli species, including approximately 2000µg/mL of proteins as was detected by a BCA assay in the current study. Such factors include beef & yeast extracts, of which absolute components are not disclosed by the manufacturer (BD Bioscience Inc). Other components include factors that help selective proliferation of lactobacilli strains such as polysorbate 80, sodium acetate, and magnesium sulphate. These molecules have been individually studied in various systems on immune regulation. Polysorbate 80 (PS80) is a surfactant and emulsifier, ubiquitously used in food preparation, medical, and laboratory settings. Its exposure to reconstructed human epidermal cells had resulted in increased expression and secretion of IL-1α and reduced cell viability, but no differences in IL-8 expression. (Coquette et al., 1999) More recently, PS80 administered to mice at low concentrations was sufficient to induce low-grade inflammation and obesity-related metabolic syndromes as compared to untreated controls. (Chassaing et al., 2015) Analysis of male rats treated with PS80 demonstrated elevated levels of IL-6, IL-1, TNF-α, and IFN-γ in liver hepatocytes. (Al-thamir et al., 2013) Similarly, sodium acetate which is also present in MRS broth, induced secretion of various inflammatory cytokines such as IL-1β, IL-8, TNF-α,
and IL-6 in human gastric adenocarcinoma epithelial cells, independent of changes in cell viability and proliferation. (Sun et al., 2005) Magnesium sulfate (MgSO₄), which helps the growth of lactobacilli, has been vastly studied in vitro to decrease inflammation, and is commonly used in clinical obstetric practice as a tocolytic for PTB as well as a treatment for women with preeclampsia to prevent seizures. Preeclamptic placentae also secrete high levels of TNF-α and IL-6, which can be suppressed by MgSO₄ in both humans and mice. MgSO₄ thus, has also been associated with neuroprotective abilities to the developing fetus. (Sugimoto et al., 2012; Chen et al., 2015) Furthermore, when myometrial tissues from term women undergoing elective cesarean sections were exposed to MgSO₄, an elevated contractility and increased intracellular calcium concentrations was detected (Fomin et al., 2006).

The increase in IL-6 secretions by myometrial cells in an in vivo intrauterine environment can be concerning for fetal development. Rat fetuses are most susceptible to maternal IL-6 during mid-gestation, (Dahlgren et al., 2006) and abnormally high concentrations of IL-6 can cause hypertension, insulin resistance, and dysregulation of the HPA-axis in adult offspring, as well as adverse outcomes in fetal neurodevelopment. (Dahlgren et al., 2001; Samuelsson et al., 2004) High maternal IL-6 in pregnant women has recently been associated with larger right amygdala volume and higher amygdala interaction with various brain regions in offspring, which were together associated with reduced impulse control by infants at 24 months. (Graham et al., 2017) In rodents, increased IL-6 due to maternal immune activation has also been identified as a key mediator in behavioural, histological, and genetic abnormalities resembling schizophrenia and autism, as mice with IL-6-/- mice or mice treated with anti-IL-6 antibody did not demonstrate these alterations. (Smith et al., 2007) Moreover, increased maternal IL-6 concentrations have been associated with increased risk of cerebral palsy, (Yoon et al., 2003)
increased fetal adiposity, (Radaelli et al., 2006) and altered neurodevelopmental structures and hormonal axes. (Dahlgren et al., 2006) In the current study, IL-6 secretions were induced by GR1SN-MRS alone, which highlights concerns for the developing fetus in a \textit{in vivo} environment that should be considered when translating these studies to \textit{in vivo} or clinical settings. Current knowledge regarding offspring outcomes from \textit{in vivo} administration of lactobacilli cultures (live or supernatant) is limited to fetus viability, birth and placental weight data, and has not extended to examination of neural deficits, which may be an important factor to consider in future \textit{in vivo} studies examining the effects of lactobacilli during gestation. Contrastingly, a recent mouse study examining the \textit{in vivo} effects of systemic GR1SN-MRS administration followed by local LPS stimulation detected a significant suppression of IL-6 and other cytokines in the myometrium, placenta, amniotic fluid, and maternal plasma, (Yang et al., 2014) demonstrating differential effects of GR1SN-MRS dependent on the mode of administration. \textit{In vitro} macrophage studies demonstrate that anti-inflammatory molecules such as IL-10 and G-CSF are produced in response to GR1SN-MRS treatment. (Kim et al., 2006) This may suggest a potential mechanism by which systemic GR1SN-MRS exhibits differential cytokine expression in local gestational tissues. For instance, when GR1SN-MRS is administered systemically \textit{in vivo}, increased anti-inflammatory cytokine secretion by immune cells may regulate local immune states in the uterus.

\textbf{4.2 In vivo functional discrepancies of GR1SN effect}

Contradictory to my original hypothesis, I found that prophylactic administration of IP-GR1SN was not able to prevent the onset of PTB induced by systemic LPS in pregnant CD-1 mice. Here, I suggest three potential explanations: (1) the mode of LPS administration between local IU and systemic IP infusions, (2) the tissue specificity of GR1SN, and (3) the composition of GR1SN-PBS as compared to GR1SN-MRS.
4.2.1 Intrauterine vs. intraperitoneal mode of LPS infusion

Previously, it was observed that IP injections of GR1SN-MRS resulted in 43% reduction of PTL induced by IU-LPS administration (Yang et al., 2014). However, in the present study, IP administration of neither GR1SN-MRS nor GR1SN-PBS inhibited PTB induced by IP-LPS (Table 4).

Table 4. Summary of current and previous in vivo results showing rates of LPS-induced PTB following prophylactic GR1SN treatment

<table>
<thead>
<tr>
<th>Study</th>
<th>Pre-treatment</th>
<th>LPS</th>
<th>Difference in rates of PTB (%)</th>
<th>Significance (P&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Yang et al. 2014)</td>
<td>GR1SN-MRS (IP)</td>
<td>LPS (IU)</td>
<td>43%</td>
<td>Yes</td>
</tr>
<tr>
<td>Current</td>
<td>GR1SN-MRS (IP)</td>
<td>LPS (IP)</td>
<td>12%</td>
<td>No (P=0.4)</td>
</tr>
<tr>
<td></td>
<td>GR1SN-PBS (IP)</td>
<td>LPS (IP)</td>
<td>n/a</td>
<td>No (P=0.58)</td>
</tr>
</tbody>
</table>

The mode of LPS administration is an obvious variable in in vivo experiments. The previous study by Yang et al. focused on the ability of GR1SN-MRS administered intraperitoneally to attenuate the effects of local, IU LPS infusion, which was performed by mini-laparotomy procedures. In the present study, I tried to extend these effects to observe GR1SN-mediated attenuation of immune responses following systemic LPS administration. However, there are two influential variables to be considered following surgery that are absent in the current study; wound healing and surgical stress response.

Wound healing is a long-term process starting with blood coagulation, inflammation, re-epithelialization, and ending with matrix and tissue remodelling. Inflammation is an early wound healing response element, during which TGF-β functions as a pro-inflammatory mediator to promote leukocyte recruitment and activation by inducing secretion of IL-8, MCP-1, TNF-α, IL-1β, and platelet derived growth factor (PDGF) in an autocrine manner. This pro-inflammatory
response and leukocyte recruitment peaks 24 to 48 hours post-wound, (Leibovich and Ross, 1975; Koh and DiPietro, 2011) after which TGF-β regains its anti-inflammatory characteristic and promotes production of anti-inflammatory factors such as IL-4. (Wahl, 1994)

Conversely, surgical stress is the systemic response that causes immunosuppression and additional perturbations in genomic, metabolic, and hormonal regulation following surgical injury. Glucocorticoid, an upregulated hormone in response to surgical stress, is a known mediator of anti-inflammatory signaling, which can repress wound healing and expression of pro-inflammatory factors. Furthermore, glucocorticoid activity has been demonstrated in relevance TLR regulation to synergistically induce TLR2 expression with TNF-α and IL-1β, (Hermoso et al., 2004; Sakai et al., 2004; Shibata et al., 2009) suggesting a potential role in TLR2-mediated endotoxin tolerance. Interestingly, a recent study elucidated the role of glucocorticoids in endotoxin tolerance in its ability to upregulate miRNA-511-5p expression in human monocytes which directly reduces TLR4 signaling activity, and downstream production of pro-inflammatory cytokines (Curtale et al., 2017). Together, the systemic responses induced by wound healing and surgical stress add an additional layer of anti-inflammatory signaling through TGF-β and glucocorticoids, which may have contributed to immune suppression in pertinence to the delay of PTB induced by IU-LPS.

In addition to the absence of such events that promote anti-inflammatory signaling following surgery, we can further speculate that the effect of GR1SN in the amount administered is insufficient to suppress the effects of IP-LPS. IP-LPS activates systemic inflammation, which induces a much stronger inflammatory response than IU-LPS through activation of peripheral leukocytes and adjacent tissues in addition to local uterine tissue and its resident leukocytes. Previous studies have demonstrated that murine macrophages treated with GR1SN demonstrated
preferential production of G-CSF in extremely high concentrations (Meshkibaf et al., 2015), which promotes suppression of LPS-induced TNF-α expression in adjacent cells in a paracrine manner (Kim et al., 2006). Therefore, it can be suggested that GR1SN in the amount administered cannot prime all macrophages that will later be stimulated by LPS to promote G-CSF production.

4.2.2 Tissue specificity of Lactobacilli effect

In section 3.6, I demonstrated that GR1SN contains unique factors not present in SN of other commensal lactobacilli (L. rhamnosus GG, L. lactis, L. casei, L. reuteri RC-14) that suppresses LPS-induced cytokine secretions by myometrial cells in vitro, thus indicating tissue-specific effects of lactobacilli SN. In vivo, IP injection of GR1SN affects many more cell types than solely myometrial cells. Previous in vitro studies have elucidated the anti-inflammatory effect of GR1SN in human trophoblasts (Yeganegi et al., 2010), decidual cells (Li et al., 2014), amniotic cells (Koscik et al., 2017), and murine macrophages (Meshkibaf et al., 2015). However, the effect of L. rhamnosus GR-1 in cytokine regulation in other tissue types such as the intestine are limited. One study reported resolved diarrhea, flatulence, and nausea in 12 HIV/AIDS patients who took oral supplements of L. rhamnosus GR-1 and L. reuteri RC-14 for 15 days, (Anukam et al., 2008) while another study examining the effects of oral L. rhamnosus GR-1 and L. reuteri RC-14 supplements for 14 days in healthy women found no changes in serum IgG, IgA, or IgM, slight increases in IL-6 and IFN-γ that remained within normal ranges, and undetectable levels of IL-2 and IL-4 (Gardiner et al., 2002). Therefore, it can be speculated that GR1SN does not elicit anti-inflammatory effects or induce endotoxin tolerance in all tissues, resulting in persistent high cytokine secretions following IP-LPS administration and eventual PTB.
4.2.3 Compositional differences between GR1SN-MRS and GR1SN-PBS

Although both GR1SN-MRS and GR1SN-PBS did not delay PTB induced by systemic LPS, differences in GR1SN composition should be considered for future experiments. In the current study, BCA assays of MRS broth and GR1SN-MRS demonstrated 2000 and 4000µg/mL of protein respectively, allotting for approximately 2000µg/mL of protein secreted from L. rhamnosus GR-1. Conversely, only 22µg/mL of protein were quantified in GR1SN-PBS, roughly 100 times less protein than GR1SN-MRS. Reviewing the definition of probiotics, “live microorganisms which when administered in adequate amounts, confer a health benefit on the host” (FAO and WHO, 2001), I speculate that there was not sufficient quantity of active components in GR1SN-PBS required to elicit benefits to pregnant mouse. GR1SN-MRS was collected after a 12-hour incubation period, which was required to culture bacteria from lag to exponential phase while GR1SN-PBS was collected after only 2 hours of incubation to maintain the bacterial growth in exponential phase. The difference in incubation times may likely contribute to the difference in protein quantity.

Additionally, the change in incubation medium from MRS to PBS may have also resulted in altered protein content. MRS broth contains various factors and nutrients critical for the growth of lactobacilli strains. It is conceivable that in the absence of these growth factors, GR-1 metabolites change, and that GR1SN-PBS contain different metabolic components than those found in GR1SN-MRS. More importantly, growth in PBS starves lactobacilli which could lead to a selective culture whereby only bacteria with mutations to survive nutrient-depleted environments proliferate, similar to how bacteria grow antibiotic resistance. (Watson et al., 1998; Nguyen et al., 2011) Starvation of Lactobacillus casei modeled by 0% lactose in growth medium was shown to suppress catabolic pathways of lactose and galactose, as well as nucleotide and protein synthesis. (Al-Naseri et al., 2013) Similarly, a study of Lactobacillus brevis
demonstrated alterations in glucose and amino acid catabolizing pathways, inducing stress-response mechanisms. (Butorac et al., 2013) Interestingly, Lactobacillus brevis starvation also demonstrated that the pH of cultured medium increased after prolonged bacterial starvation due to decreased acid production, which may indicate altered effects of bacterial SN. (Butorac et al., 2013)

Overall, active components in GR1SN that delay the onset of LPS-induced PTB in vivo and suppress LPS-induced inflammatory responses in vitro have yet to be determined. Through my work, I have suggested a potential mechanism of GR1SN-mediated endotoxin tolerance dependent on the TLR2 receptor. I have emphasized the specificity of host-microbiome interactions by demonstrating GR1SN as a unique prophylactic agent capable of suppressing LPS-induced cytokine secretions in vitro. My in vivo work suggests that prophylactic GR1SN administration cannot offer protection against systemic inflammation, and that an adequate amount of active component may be required to elicit beneficial effects.

4.3 Future Directions

My current in vitro work demonstrates LPS-induced cytokine suppression by Pam3CSK, a synthetic triacylated lipoprotein, that mimics the amino end of bacterial lipoproteins. From this, I speculate that the active component of GR1SN may be a triacylated lipoprotein. To test this hypothesis, L. rhamnosus GR-1 could be cultured with globomycin, an inhibitor of the lipidation process in lipoprotein synthesis. In the event that GR1SN derived from cultures with globomycin fail to elicit the suppression of LPS-induced cytokines as was observed in my study, this may confirm the functional capabilities of bacterial lipoproteins.

We demonstrated previously that inflammation is a key driver of uterine contraction. The involvement of inflammatory pathways in labour involves the production of other labour-
associated molecules such as PGs, MMPs, or OXTR, in addition to increased expression of cytokines. Thus, it may be interesting to observe how these molecules are influenced by GR1SN pre-treatment. Furthermore, work by Hutchinson et al. demonstrates that there may be an involvement of an independent Rho/ROCK pathway to directly induce myometrial contractions. It is interesting to observe if GR1SN can influence this pathway in the myometrium. (Hutchinson et al., 2014)

Previous work from our lab has demonstrated that static myometrial stretch was a potent inducer of various inflammatory markers including pro-inflammatory cytokines including IL-6 and IL-8. Thus, it may be interesting to investigate whether the positive effects of GR1SN pre-treatment remains limited to mechanisms of endotoxin tolerance, or if its effects are more global in regulating cytokine suppression linked to the onset of labour.

In vivo, several experiments could test this possibility. First, it would be useful to test whether prophylactic GR1SN-PBS could prevent the onset of local LPS-induced PTL. This would more closely resemble previous work by Yang et al. that demonstrated prophylactic GR1SN-MRS as able to prevent intrauterine LPS-induced PTL. Furthermore, I had hypothesized that the ineffectiveness of GR1SN-PBS in vivo may be attributed to insufficient quantities of active component of GR1SN-PBS, which contained 100 times less protein than GR1SN-MRS used by Yang et al. Thus, it will be important to test concentrated forms of GR1SN-PBS which match the protein concentrations found in GR1SN-MRS.
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