LEUKOCYTE MIGRATION AND PARTURITION

by

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Abstract

Parturition is a complex phenomenon that is regulated by leukocyte invasion, progesterone withdrawal, and local inflammation. Of these regulatory mechanisms, leukocyte invasion is of interest for diagnostic development. Leukocyte invasion is thought to be mediated by an increase in output of chemotactic factors (chemokines) from intrauterine tissues and an enhancement of peripheral leukocytes to respond to chemotactic signals. Once these leukocytes are recruited from the plasma to the uterine tissues where they become anchored, they release an array of inflammatory mediators that amplify this response thereby facilitating uterine transition from pregnancy to parturition, cervical softening, and fetal membrane breakdown, which culminates in expulsion of the fetus and initiation of uterine involution.

Leukocyte invasion is therefore a signal for parturition, and it has been studied previously using a tool called the Leukocyte Migration Assay (LMA). Our lab has previously demonstrated that the LMA can predict labour onset within 7 days. I streamlined the LMA and reduced the time and cost required to perform the assay by over 60% and 80% respectively, making it more feasible for clinical implementation. The streamlined LMA was also used to study the mechanisms that regulate leukocyte invasion during normal and preterm labour. In our study, intrauterine administration of IL-1β was used to induce preterm birth (PTB) in mice.

We determined that mouse gestational chemotactic factors (GCF) in the lower uterus peak just prior to labour, coinciding with the timing of leukocyte invasion. This increase in GCF was not detected during PTB despite observing a greater density of neutrophils in the lower uterus. Induction of PTB resulted in the increased abundance of proinflammatory cytokine mRNA in peripheral leukocytes, as well as the enhanced responsiveness of leukocytes to respond to chemokines. This suggests that IL-1β mediates leukocyte invasion by enhancing leukocyte

chemotaxis and not by increasing GCF secretion. Moreover, we demonstrated that in humans, IL-1β does not enhance leukocyte chemotaxis directly, but likely stimulates the secretion of an intermediary priming factor from the fetal membranes. Our finding that leukocytes can be enhanced for chemotaxis artificially through incubation with maternal serum from term labouring women suggests that the intermediary priming factor may be released into circulation where they may exert a priming effect on peripheral leukocytes.

In addition, we studied whether 101.10 (aka Rytvela), a novel allosteric modulator of the IL-1β receptor and potential therapeutic for PTB, could reverse IL-1β-mediated PTB, neutrophil infiltration of maternal and fetal tissues, leukocyte priming, and detection of proinflammatory cytokine mRNA in peripheral leukocytes. These effects of IL-1β in the mouse were successfully reversed with 101.10 co-treatment.

The LMA forms the basis of a clinical diagnostic test that estimates the time until the spontaneous labour onset, whether term or preterm. Furthermore, our results suggest that the LMA can be used to assess the level of maternal, and by proxy, fetal, inflammation during pregnancy. Additionally, the LMA can be used to assess the degree of inflammatory suppression that is provided by anti-inflammatory inhibitors, including 101.10. Combined, our findings offer new opportunities for diagnosing, treating and assessing the efficacy of treatment of maternal and fetal inflammation. They may also lead to new and important questions about the origins and effects of maternal and fetal inflammation.

Preface

This thesis is an original work by Han Lee. The research project, of which this thesis is a part, received ethics approval from the University of Alberta Research Ethics Board. No part of this thesis has been previously published.

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Abbreviation Table

AP	Activating Protein
BPD	Bronchopulmonary Dysplasia
CF	Chemotactic Factor
CVF	Cervicovaginal Fluid
CX	Cervix
Cx-43	Connexin-43
DAMP	Damage Associated Molecular Pattern
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
ER	Endoplasmic Reticulum
FFN	Fetal Fibronectin
FM	Fetal Membrane
GCF	Gestational Chemotactic Factor
GD	Gestational Day
GI	Gastrointestinal
hL	Human Leukocytes
IL	Interleukin
IRAK	Interleukin-1 Receptor Associated Kinase
LPS	Lipopolysaccharide
MLCK	Myosin Light Chain Kinase
MMP	Matrix Metalloprotease Protein
NEC	Necrotizing Enterocolitis
NF	Nuclear Factor
OT	Oxytocin
PAMP	Pathogen Associated Molecular Pattern
phIGFBP1	Phosphorylated Insulin-Like Growth Factor Binding Protein-1
PG	Prostaglandin
PL	Placenta
PTB	Preterm Birth
PTNL	Preterm Not in Labour
RDS	Respiratory Distress Syndrome
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SP-A	Surfactant Protein A
Syk	Spleen Tyrosine Kinase
TIMP1	TIMP metallopeptidase inhibitor 1
TL	Term Labour
TNF	Tumor Necrosis Factor
TNL	Term but not in Labour
UAP	Uterine Activation Proteins
LU	Lower Uterus
UU	Upper Uterus

Chapter 1. General Introduction

Parturition is the physiological phenomenon that expels the fetus from the uterus, thereby marking the end of pregnancy and setting involution into motion. Through involution, the gestational organs are returned to their normal pre-pregnant state, and this process is especially prominent in the uterus, which has undergone tremendous growth in size to accommodate the developing fetus during pregnancy [1]. The mechanisms that initiate and regulate parturition are therefore not only important for birth, but also for enabling the mother to participate in another pregnancy. Previously-studied regulatory mechanisms of parturition include immunological control via leukocyte invasion and hormonal control via systemic and functional progesterone withdrawal. Both mechanisms will be discussed and used to describe the current model for the regulation of parturition and identify where the knowledge gap lies.

1.1. Immunological Control of Parturition. Parturition can be initiated by both sterile [2] and infectious inflammatory stimuli, leading to a phenomenon called leukocyte invasion, whereby leukocytes infiltrate the gestational tissues as a woman prepares to undergo parturition. The phenomenon of leukocyte invasion is conserved among several species including humans, rats, and guinea pigs [3-5]. In humans specifically, leukocyte invasion has been detected in the term laboring (TL) myometrium [6], term cervix [7], and TL decidua [8]. During parturition, these tissues are important respectively for physically contracting and expelling the fetus, dilating and facilitating fetal passage, and forming the maternal part of the placenta where detachment occurs [2, 8]. Once anchored, these leukocytes release an array of matrix metalloproteases (MMPs), prostaglandins (PGs), cytokines, chemokines and other effectors that not only amplify this

inflammatory event, but also facilitate the uterine transition from pregnancy to labor, remodeling of the cervical extracellular matrix (ECM), and breakdown of the fetal membranes (chorion, amnion), which culminate in parturition [9].

To discuss how the immune system regulates parturition in more depth, it is easiest to explain starting at the end and work backwards. In the following sections I will detail what is known about the effectors that are released by invading leukocytes and the surrounding tissues to exert pro-labor effects, which are called uterine activation proteins (UAPs), as well as the cytokines and chemokines that are released. Then, I will describe how the various subpopulations of leukocytes contribute to the production of these effectors, and finally I will explain how they might be recruited to these gestational tissues.

1.1.1. Uterine Activation Proteins. Before labour begins in humans and other animals, the uterus undergoes a transition to become capable of fetal expulsion. The key molecules involved in this process are UAPs, proteins that share four key traits: they 1) experience a change in expression during labour that is reversed post-partum, 2) have contractile effects on the uterus that are dependent on gestational age, 3) can be induced by pro-inflammatory cytokines, and 4) contribute to labour [10]. For example, gap junctions are formed between myometrial cells to facilitate the transmission of contractile signals, and increased expression of myometrial OT receptors sensitizes uterine smooth muscles to OT, a key hormone that stimulates myometrial contraction [11, 12]. Moreover, the upregulation of calcium channels allows for the increased influx of calcium into myocytes [12]. Two key UAPs include MMPs and PGs.

MMPs are a family of zinc-dependent proteases that are secreted into the extracellular matrix (ECM) as proenzymes that can be activated by proteolytic cleavage [13]. MMP activity is

tightly regulated by complex interactions between proteolytic activators, tissue inhibitors of metalloproteinases (TIMP), and other activators and inhibitors [13]. One of MMP's role during parturition is to promote fetal membrane rupture by degrading the ECM and weakening the structure of the FM, and elevated MMP in the amniotic fluid has been correlated with a decline in membrane tensile strength [13]. Moreover, accumulation of MMP9 at the human fetal membranes has been observed directly during and after labor, and higher MMP2 and MMP9 activities have been detected during both term and preterm labor [14, 15]. Another role of MMPs is to remodel the cervix from a closed and firm state that prevents immature passage of the fetus, to an elastic and flexible state that can facilitate fetal passage; this function was evidenced by >6x greater concentrations of MMP9 at >6 cm dilation than at >2 cm dilation and by increased activity of MMP9 during term labor [13, 16]. A final role for MMPs has been described in postpartum uterine involution when it plays an important role in degrading the collagen in the ECM to return the uterus to its pre-pregnancy state [13].

On the other hand, PGs are involved in parturition through five distinct but integrated physiological events. First, it can facilitate membrane rupture by stimulating MMP activity and inducing cell apoptosis [17, 18]. Second, a large body of evidence illustrates the role of PG in cervical ripening and dilatation, one mechanism of which is through the stimulation of MMP activity [19, 20]. Third, PGs also stimulate myometrial contraction directly as demonstrated by exogenous application of PG *in vivo* and *in vitro* [20, 21]. Fourth, PG plays a role in placental separation [20]. Finally, uterine involution is closely associated with plasma levels of $PGF_{2\alpha}$ in cows and sheep, and administration of $PGF_{2\alpha}$ is sufficient to promote uterine involution in cows [20].

These UAPs, among others, can be induced by various inflammatory cytokines produced by the invading leukocytes and surrounding tissues.

1.1.2. Inflammatory Cytokines. A major cytokine that has been suggested to play a central role in parturition is IL-1β, an inflammatory mediator that has generally been characterized as a player in host-response and resistance to pathogens. The cytokine is expressed as the inactive 31 kDa precursor, pro-IL-1β, in response to the binding of a pathogen or damage associated molecular pattern (PAMP and DAMP) to a pathogen recognition receptor (PRR) that is present on the cell membrane or in the cytosol (Fig. 1A). An additional PAMP or DAMP encounter induces caspase-1 activation via recruitment to the inflammasome: a molecular scaffold composed of adaptor molecules, cytosolic pattern recognition receptor, and pro-caspase-1 [22]. Caspase-1 then cleaves pro-IL-1β to active IL-1β. In neutrophils, the production of IL-1β is not exclusively dependent on caspase-1; other proteases may compensate for the loss of caspase-1 [23].

Unlike IL-1β synthesis, the mechanism of IL-1β secretion from the cell is unclear. Conventional protein secretion uses the endo-membrane system comprised of the endoplasmic reticulum (ER) and Golgi apparatus, to export proteins outside the cell or to specialized sub-cellular organs [24] (Fig. 1B). Co-translational translocation begins with a signal recognition particle (SRP) which detects and binds a signal sequence at the N-terminus of a nascent peptide as it emerges from the ribosome [24]. The SRP then binds its receptor on the ER, enabling translocation of the nascent peptide chain to the ER lumen through the protein-conducting channel formed by sec61α and sec61γ [25]. In the ER lumen, chaperones help the protein fold correctly, upon which the protein becomes packaged in coat protein complex II apparatus (COPII)-coated vesicles that fuse with and deposit the cargo protein into the Golgi apparatus

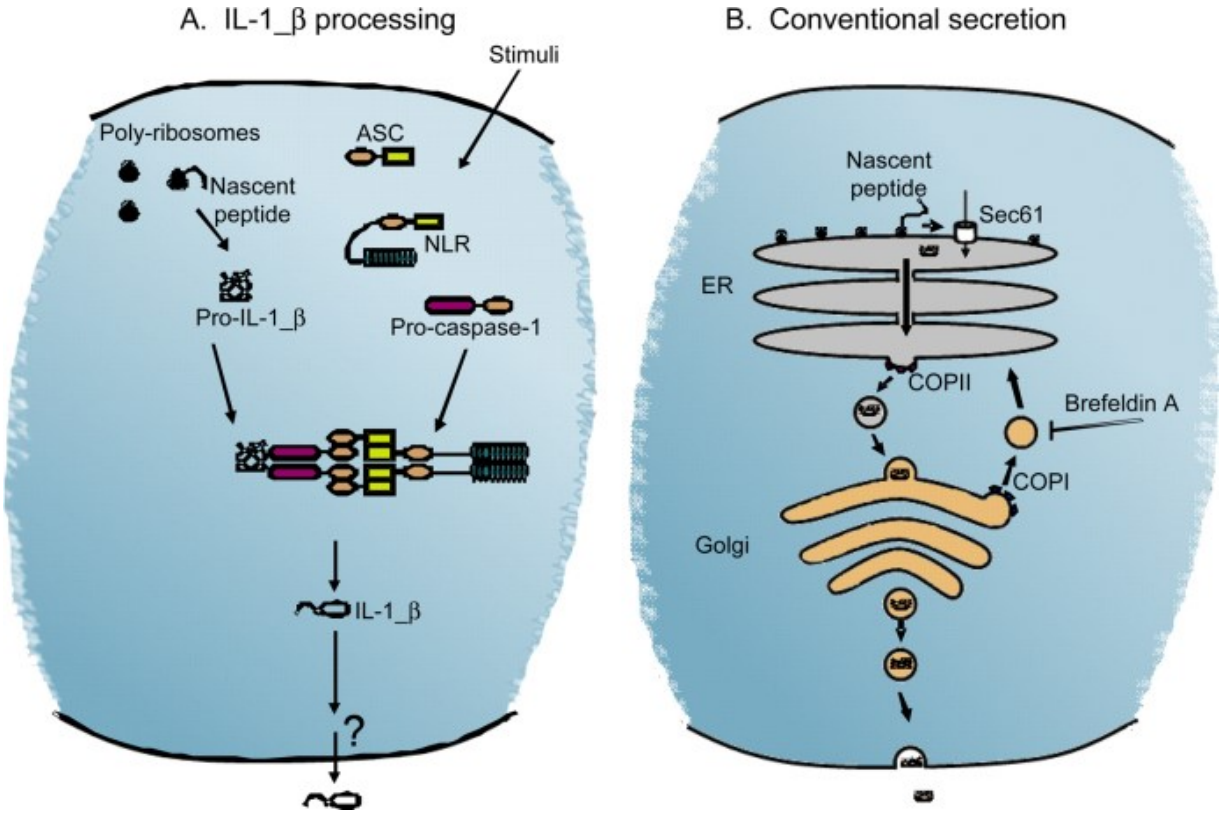

Figure 1. *Schematic diagrams showing the components and the formation of the inflammasome, and of the conventional pathway of protein secretion*, as published in Lopez-Castejon G and Brough D, 2011 [81].

[26]. The cargo protein undergoes a series of secondary modifications before it is packaged and transported to its final destination [27].

The reason why IL-1β does not follow conventional secretion is because it lacks a signal peptide [28]. This is supported by immunoelectron microscopy evidence that confirms the absence of IL-1β from both the ER and Golgi apparatus in LPS-activated monocytes [29]. IL-1β is instead translated on free ribosomes in the cytosol, where it remains until secretion [30].

From there, however, the mechanisms of IL-1β secretion are not well understood. A fraction of IL-1β can be released via the shedding of microvesicles from the plasma membrane and the secretion of exosomes [31, 32]. IL-1β can also be released via pyroptosis of the IL-1β-expressing cell, as is observed in macrophages following infection by NLRC4-activating pathogenic bacteria [33]. During pyroptosis, an infected macrophage simultaneously kills itself and releases inflammatory cytokines, creating a hostile environment for pathogenic growth [33]. Recently, necrosis of IL-1β-producing cells driven by signal II agents like ATP, nitroglycerin, stretpolysin O, uric acid crystals, and alum salts has been identified as a mechanism of IL-1β-secretion [34]. Moreover, ATP binding cassette transporter A1 has been demonstrated to contribute to the secretion of IL-1β in macrophages [35].

Once secreted, IL-1β exerts its effects by binding to a primary IL1 family receptor subunit such as IL1RI, IL18Rα, IL1 Rrp2, or ST2, which subsequently recruits an accessory receptor like IL1 receptor accessory protein (RAcP) or IL18Rβ to mediate downstream signaling events [36]. Specifically, IL-1β-binding initiates IL1RI binding to the adaptor protein Myeloid differentiation primary response gene 88 (MyD88) that activates IL1R-associated kinase 4 (IRAK4) [37]. In an inactive cell, an adaptor protein such as a Toll interacting protein forms a complex with IRAK1 and inhibits IL1 signaling by blocking IRAK1 phosphorylation; in a

stimulated cell, IL-1β-activated IRAK4 phosphorylates and activates IRAK1 [38]. IRAK1 then associates with TNF receptor-associated factor 6 to initiate two distinct signaling cascades that induce either the nuclear factor (NF)-κB or activating protein (AP)-1-dependent expression of proinflammatory cytokines and mediators [36]. AP-1 has been suggested to play an important role in labour, and its activation or inhibition is sufficient to induce or delay labour in mice respectively [39, 40].

By extension, the trigger for AP-1 activity, IL-1β, may play a key role in the parturition of humans and other animals. In further evidence of this, IL-1β administration alone elicits labour in several animal models including the mouse and non-human primate, and inhibition of the IL-1β receptor prevents IL1-induced preterm birth (PTB) in the mouse [41-43]. In the human, elevated blood levels of IL-1β, and polymorphisms of *IL-1β* and *il1rα* are associated with PTB [44, 45]. IL-1β stimulates UAP expression, and more so in the presence of $PGF_{2\alpha}$ [46, 47]. IL-1β can also improve neutrophil survival in the presence of peripheral blood mononuclear cells, and mediate neutrophil recruitment via the upregulation of e-selectin and other cellular adhesion molecules [48, 49].

Other cytokines and chemokines that may play important roles in parturition include IL-6, TNFα and IL1α. IL-6 is a 26 kDa protein that binds to IL-6Rα [50]. IL-6 increases expression of genes for PG synthesis and uterine signaling [51, 52]. Moreover, *IL-6* null mice deliver 24 hours later than the wild type [53]. A function for IL-6 in parturition is evidenced by the association between the concentration of IL-6 in amniotic fluid and gestational tissues, and the onset of normal and preterm labour [54-56]. Furthermore, loss-of-function polymorphisms of *IL-6* protect against PTB, whereas polymorphisms that increase IL-6 activity are predictive of PTB [57, 58]. Another role for IL-6 may be to enhance leukocyte chemotaxis. First, the concentration

of IL-6 in maternal serum is higher in women at TL than those at term but not in labour (TNL) [59]. Second, pre-incubation of neutrophils with IL-6 (10 or 100 ng/ml) significantly enhances chemotaxis towards IL-8 [60]. It is interesting that pre-incubation of neutrophils with IL-6 does not affect the expression of CD11b, a neutrophil activation marker which has been reported to be upregulated in PTB and TL pregnancies compared to preterm but not in labour (PTNL) and TNL pregnancies respectively [60, 61].

TNFα is a cytokine that is heavily involved in systemic inflammation and the regulation of immune cells. The concentration of TNFα is greater in the maternal decidua, amnion, chorion, isolated decidua, and amniotic fluid at TL than TNL [62, 63]. The role of TNFα in parturition is unclear, but it has been suggested that TNFα may play a role in upregulating PR-A via the NF-κB signaling pathway, resulting in a shift in the PR-A:PR-B ratio and mediating a functional withdrawal of progesterone [62]. TNFα can also upregulate PG synthesis via increased COX-1 expression [64]. Furthermore, TNFα has been reported to decrease the expression and activity of prostaglandin dehydrogenase (PGDH), the primary enzyme that catalyzes PG into inactive metabolites, in intact fetal membrane disks and cultured chorion and placental trophoblast cells [65].

IL1α, like IL-1β, is part of the IL1 family of cytokines; however, unlike IL-1β, IL1α is expressed constitutively. IL1α regulates IFNγ and its effector genes by maintaining a basal level of the transcription factor NF-κB [66]. A role for IL1α in parturition has been suggested by reports that polymorphisms of *il1α* are associated with PTB in Japanese women [67]. Furthermore, like IL-1β, IL1α peaks in cervicovaginal fluid (CVF) 4-14 days before labour [68]. IL1α is also able to elicit PTB in mice, which is reversible using IL1R antagonist [69]. Not only

does this support the importance of IL1α, but it also suggests that IL1R, which binds both IL1α and IL-1β, is likely just as important as its ligands to the regulation of parturition.

In all, the UAPs, cytokines, and chemokines play an important role in the immunological control of parturition. I will now discuss the various leukocyte subpopulations that contribute to the production of these effectors.

1.1.3. Leukocyte Subpopulations in Parturition. There is a plethora of different leukocyte subpopulations, including macrophages, granulocytes, lymphocytes, and mast cells, many of which experience an increase in density towards labor onset (Fig. 2). Of interest to parturition are the macrophages and granulocytes, and more specifically the neutrophils.

Generally, macrophages are large phagocytic leukocytes that originate from blood monocytes that leave the circulation and differentiate in various tissues. Macrophages are generally responsible for detecting and destroying harmful cells, presenting antigens to T-cells, and amplifying inflammatory signals [70]. In the maternal uterus, macrophages are present throughout pregnancy and increase in density during late gestation [6]. A role they may play in parturition is to produce MMP9, a key player in both the physiological and pathological rupture of the fetal membranes, as well as the breakdown of extracellular matrix which comprises the structure of the cervix [71]. Moreover, macrophages are a major source of cytokines including IL-1β, IL-6, IL-8, TNF-α, and many more, which, as described earlier, exert a plethora of effects that promote parturition. In addition, macrophages confer protection to the mother during and after parturition by functioning as a cellular reserve to provide functionally matured cells necessary for activation of immune responses to fetal antigens that are introduced to the maternal circulation during placental detachment [71, 72].

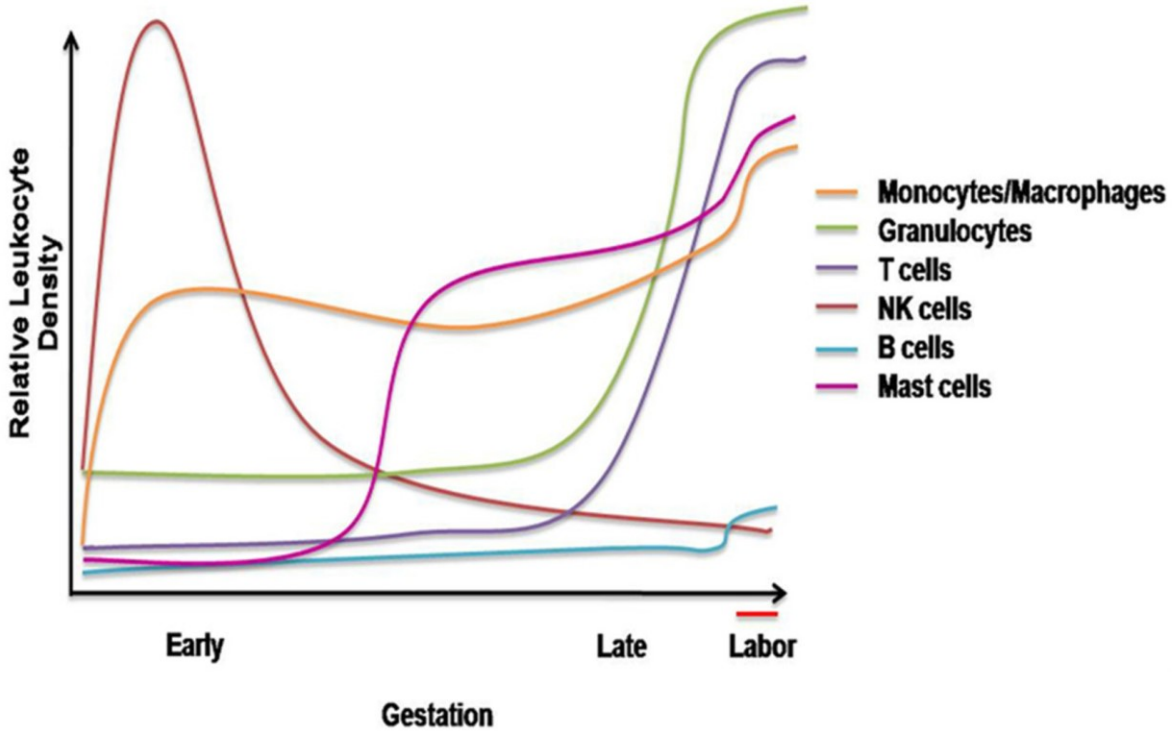

Figure 2. *Timing of presence of leukocytes in the fetal-maternal interface during pregnancy*, as published in Gomez-Lopez N *et al*, 2010 [9].

Granulocytes also infiltrate the gestational tissues during late gestation [6, 73]. Granulocytes consist of eosinophils, basophils, and neutrophils, and are classified by the presence of granules in their cytoplasm. Briefly, eosinophils combat multicellular parasites and promote allergic disorders [74], and they are recruited by antigen-exposed local Th2 cells that produce IL5 and IL13; these cytokines promote eosinophil activation and induce local cells to secrete eotaxins respectively [74]. On the other hand, basophils are effector cells of the type 2 immune response; basophils bind circulating IgE, leading to the activation of a spleen tyrosine kinase (Syk)-mediated signaling cascade that culminates in degranulation and release of IL4 and IL13, key mediators of the allergic response [75]. Our chief granulocytes of interest during labor are the neutrophils, which are sparse in the myometrium before labour and abundant during labor [6]. Neutrophils account for 50%-70% of all circulating leukocytes and comprise the greatest density of immune cells at the maternal uterus during labor [6].

Circulating neutrophils are inactive, and their activation is a key step of the inflammatory response [76]. The early stages of neutrophil recruitment begin with the partial activation of neutrophils as they transit through the vascular endothelium, followed by full activation in response to a proinflammatory stimulus in the tissue [76]. Generally, once neutrophils are activated, they participate in host defense by killing pathogens via phagocytic uptake, generating reactive oxygen species (ROS), undergoing degranulation, and releasing neutrophil extracellular traps (NETs) [76]. Excessive NET formation has been associated with preeclampsia of pregnancy and detected in the amniotic fluid of women with intra-amniotic infection [77, 78]. However, parturition and neutrophil invasion can occur even in the absence of infection, suggesting that the primary function of invading neutrophils is not host defense.

Rather, these invading neutrophils have been suggested to regulate pro-labor events. Neutrophils are a rich source of neutrophil elastase, collagenase, and MMP9, three mediators of cervical ripening and fetal membrane breakdown [79-82]. Neutrophils can also enhance the expression of MMP-1 and MMP-3 in surrounding decidual cells, and they are an abundant source of pro-inflammatory cytokines that amplify leukocyte recruitment and function [76]. Some of these pro-inflammatory cytokines stimulate the expression of cyclooxygenase 1 (COX-1) in mice and COX-2 in humans, the former which has been suggested to control the timing of parturition and the latter which is a key mediator of uterine transition by way of PG synthesis [83-85; Leimert and Olson, unpublished]. A further role for leukocytes in involution has been suggested by their involvement in postpartum repair and remodeling of the uterus and cervix [86, 87]. Neutrophils specifically are critical for the oxidative or proteolytic modification of damaged tissue that allows the phagocytosis of cellular debris during muscle regeneration by macrophages or other neutrophils [88]. To exert these effects, neutrophils must first be recruited to the target tissues through transendothelial migration (TEM).

1.1.4. Transendothelial Migration. TEM is the mechanism by which leukocytes cross the endothelial cells lining the vessels at the site of inflammation (Fig. 3) [89]. As circulating leukocytes move through the bloodstream passively via laminar flow of blood, postcapillary venules at sites of inflammation tend to constrict, leading to reduced blood flow rate and greater opportunity for leukocytes to contact the endothelial cells lining the vessel. Leukocyte adhesion molecules are expressed due to the inflammatory response at the site of inflammation, increasing the chances that these contacts will lead to effective binding. Transient binding interactions of selectins to their ligands allow the rapidly moving leukocytes to be captured from the

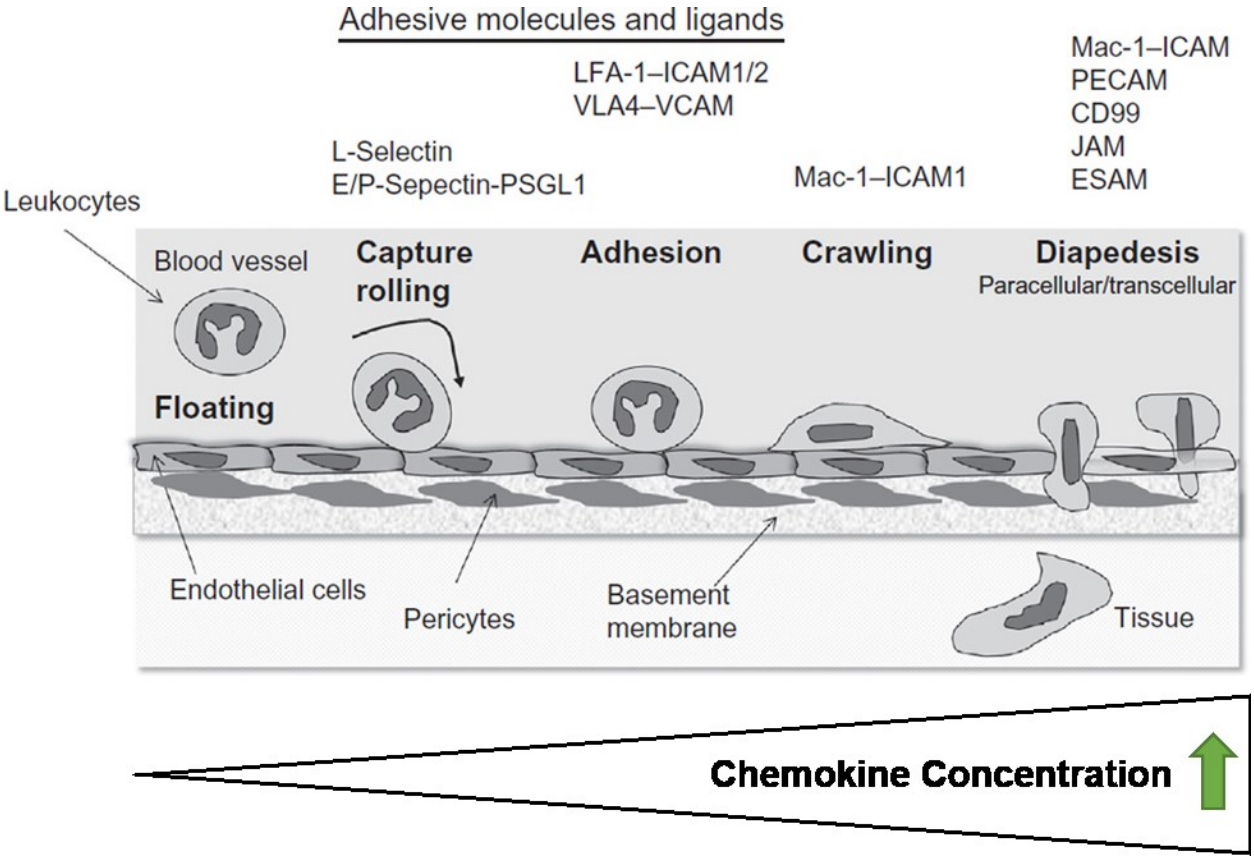

Figure 3. *The leukocyte extravasation cascade is controlled by sequential adhesive interactions between leukocytes and endothelial cells*, as published in Fillipi MD, 2016 [31].

bloodstream and tentatively bind to the endothelium. This tentative binding allows leukocytes to be activated by chemokines, which induce integrin activation. Integrins are a family of heterodimeric adhesion receptors that are inactive in their resting state. Once adhered to the endothelium, leukocytes crawl to nearby endothelial borders, mediated by Macrophage-1 antigen (Mac-1/CD11b/CD18) interacting with intracellular adhesion molecule 1 (ICAM-1/CD54) [90, 91]. Finally, leukocytes undergo diapedesis in ameboid fashion.

The key proteins involved in this process include ICAM-1, which firmly adheres leukocytes to the apical surface of endothelial cells through interactions with CD11a/b. Vascular cell adhesion molecule 1 (VCAM-1) facilitates the clustering and engagement of adhesion molecules and can stimulate intracellular calcium release to loosen the endothelial adherens junction. Junctional adhesion molecules are concentrated at endothelial cell borders and can bind to lymphocyte function-associated antigen 1 (LFA-1) on leukocytes [92]. Platelet endothelial cell adhesion molecule (PECAM-1/CD31) is an immunoglobulin that is present on both leukocytes and endothelium and is essential for TEM [93]. CD99 is similarly required for TEM of neutrophils, but is relevant during diapedesis, albeit later than PECAM [94]. Vascular endothelial cadherin (VE-cadherin) is the major adhesion molecule of the endothelial adherens junction, and it impedes TEM.

The unified model of paracellular migration describes how these proteins work together to facilitate diapedesis. LFA-1 preferentially binds ICAM-1 dimers, which initiates the clustering of ICAM-1. This in turn stimulates the phosphorylation of cortactin, which further enhances the clustering of ICAM-1 and results in the enrichment of ICAM-1 around tightly adherent leukocytes. Multimerization of ICAM-1 also leads to an increase in cytosolic calcium as well as the activation of RhoA. Meanwhile, leukocytes expressing very late antigen 4 (VLA-4) binds

VCAM-1 on endothelial cells. The clustering of VCAM-1 stimulates an increase in cytosolic calcium, activates Ras-related C3 botulinum toxin substrate 1 (Rac-1), and results in the production of ROS [95, 96]. These ROS activate protein kinase Cα, which loosens endothelial junctions. Both ICAM-1 and VCAM-1 signaling results in the phosphorylation of VE-cadherin, dissociating it from the actin cytoskeleton, subjecting it to clathrin-mediated endocytosis, and weakening the endothelial junctions. Moreover, the increase in cytosolic free calcium activates myosin light chain kinase (MLCK), inducing tension in the endothelial cells. The activation of RhoA enhances MLCK activation. Ultimately, the endothelial cells contract and expedite passage of the leukocytes.

1.1.5. Neutrophil Chemotaxis. The directionality of neutrophils is an independent process from movement itself. In response to an inflammatory stimulus, chemoattractants are released at the site of inflammation that can induce rapid changes to neutrophil shape, resulting in cell polarization that allows the cell to orient in space and migrate up a chemical gradient towards the site of inflammation [97].

Encountering a chemoattractant stimulates the corresponding G protein-coupled receptor and activates a trimeric G protein complex, which releases a $G_{\beta\Delta}$ heterodimer from inhibition by $G_{\alpha i}$ and initiates a signaling cascade [98]. This cascade results in the uniform distribution of certain chemoattractant receptors, such as cAMP receptors, on the cell membrane, and the asymmetrical localization of others, such as CCR5, to the leading edge [99, 100]. Signaling molecules involved in this process include the phosphoinositide 3-kinase (PI3K) product, phosphatidylinositol 3,4,5-triphosphate (PI[3,4,5]P3), and active Rac, both of which are asymmetrically distributed internally. This asymmetric distribution of PI[3,4,5]P3 is partially

attributed to the asymmetric distribution of PI3Kγ, a class of PI3K that binds and is activated by the $G_{\beta\Delta}$ heterodimer. The importance of PI3Kγ has been evidenced using PI3Kγ selective inhibitors to prevent neutrophil chemotaxis [101]. Overall, the process leading to the asymmetric organization of receptors is called the 'frontness' pathway.

The frontness pathway alone cannot account for how neutrophils can polarize in a uniform concentration of attractants or prevent pseudopods from forming laterally as they move forward. The 'backness' pathway is described by Xu *et al* [102]. Evidence of a backness pathway has arisen from experiments with pertussis toxin, which completely blocks frontness pathway responses: using treated neutrophil-like differentiated human leukemia-60 cells, investigators have demonstrated that chemoattractants induce morphological backness in a well-defined uropod-like structure via a signaling pathway independent of G_i. It was suggested that the backness pathway is initiated by trimeric G proteins G_{12} and G_{13}, and its downstream signaling elements are spatially organized to the back and sides of polarized neutrophils. These signaling elements include PRG (PDZRhoGEF, a Rho-specific GEF), Rho, p160-ROCK (a Rho-dependent Kinase), phosphorylated myosin light chain, and consequent activation of myosin II. The frontness-backness model proposes that actin polymers and actin-myosin assemblies induce spatial separation of the assemblies themselves to opposite sides of the cell (protrusive polymers at the front; contractile actin-myosin complexes at the back).

Once the neutrophil is polarized, four steps mediate the multiple cycles of attachment and detachment that generate neutrophil forward movement [98]: 1) the leading edge protrudes pseudopod(s) by actin flow; 2) the protruding membrane and surface receptors interact with the substrate; 3) interactions activate actomyosin contractile machinery to contract the cell body mid-region; and 4) this contraction causes the rear of the cell to move forward. Additionally, the

trailing edge of the neutrophil contains CD43 and CD44, ICAM-1, ICAM-3, and β1 integrins which aids in movement [103].

1.1.6. Neutrophil Chemotactic Factors. The initial stimuli that induce neutrophil polarization and directed movement are diverse, and they include chemokines, bacterial peptides, lipid mediators, and complement proteins. A more parturition-specific discussion of gestational chemotactic factors will be discussed in Chapter 3.

IL-8 is a major neutrophil chemoattractant that is present at elevated levels in the amniotic fluid, myometrium, decidua, fetal membranes, and maternal serum of human pregnancies with signs of infection [104]. IL-8 induces the recruitment and activation of neutrophils by binding to CXCR1 and CXCR2 on the cell surface [105]. Pregnancies without signs of infection in late gestation are also characterized by elevated levels of IL-8 in the amnion [106]. As such, IL-8 may be partially responsible for the gestational infiltration of neutrophils that coincides with labour onset in humans. In rats and mice, IL-8 is not expressed [107]. Instead, the chemokines CXCL1/KC, CXCL2/MIP-2, and CXCL5-6/LIX are functional homologues of IL-8 and belong to the same major cluster of chemokines (human chromosome 4q13.3; mouse chromosome 5qE2) that are involved in neutrophil recruitment [108].

CCL2, also known as monocyte chemoattractant protein (MCP)-1, is present at elevated levels in the mouse myometrium during labour at gestational day (GD)-19 compared to late pregnancy at GD15 [109]. It was also demonstrated that CCL2 transcripts are upregulated in the human myometrium during both normal and preterm labour [110]. In addition, CCL2 plays a key role in macrophage activation by initiating the respiratory burst necessary for macrophage maturation [111]. Activated macrophages can then subsequently produce and release

inflammatory cytokines, PGs, and MMPs that facilitate labour onset. With regards to neutrophil chemotaxis, Gouwy *et al* demonstrated that a mix of 100 or 300 ng/mL of CCL2 and 5 ng/mL IL8 produces a much stronger neutrophil chemotactic response than 5 ng/mL alone, and that CCL2 by itself did not stimulate neutrophil chemotaxis [112], suggesting that CCL2 and IL8 are synergistic for neutrophil chemotaxis.

In contrast, N-formylmethionine-leucyl-phenylalanine (fMLP) is a gram-negative bacterial peptide that acts as a highly potent neutrophil chemoattractant [113]. fMLP could be relevant to parturition in that some etiologies for preterm labor are bacterial in nature, and could play a role in recruiting neutrophils to the site of inflammation. Moreover, fMLP likely plays an important role in recruiting more leukocytes to the placental detachment site which is prone to bacterial infection and contamination after parturition [114].

Lipid chemoattractants include platelet activating factor (PAF) and leukotriene B_4, both of which can induce human neutrophil migration across 3 μm pore filters alone as well as across various epithelial and endothelial monolayers [115]. In neutrophils, PAF activates mitogen-activated protein kinase (MAPk) kinase-3 and p38 MAPk to initiate chemotactic effects (116). On the other hand, leukotriene B_4 is actively secreted by neutrophils in exosomes as they migrate towards fMLP [117], and significantly amplifies neutrophil recruitment to the primary chemoattractant by selectively modulating signaling cascades involved in cell polarization as well as serving as a potent secondary gradient [118].

The complement protein C5a is an end-target chemoattractant which stimulates neutrophil chemotaxis preferentially over intermediary chemoattractants like LTB_4 [119]. C5a can also cause an oxidative burst in neutrophils and can enhance phagocytosis, release of granule enzymes and expression of adhesion molecules on neutrophils [120]. The compliment system is

generally involved in host defense and the clearance of potentially harmful cellular debris [P], but in the context of parturition, C5a has been suggested to be a key mediator for MMP9 release in cervical macrophages to soften the cervix [121] and upregulate pro-labor mediators in the gestational tissues via CD88-mediated NF-κB activation [122]. The maternal plasma concentration of C5a is increased in women with preterm labor and intra-amniotic infection, and C5a may play a role in pathophysiological pregnancies via modulation of proinflammatory cytokine, phospholipid-derived mediator, as well as ECM remodeling enzyme release at the gestational tissues [122].

1.2. Hormonal Control of Parturition. On the other hand, the endocrine system has been implicated in regulating parturition as well, though moreso in non-human animal models. Before delving into our current understanding of the hormonal control of parturition, we will discuss the reasons why we still use animal models for research today. Ethical constraints on the use of human subjects for research restricts us to *in vitro* and *ex vivo* work, limiting our ability to study how certain proteins or systems work in the context of complex human physiology. The solution to this problem has been to use 'representative' animal models. A popular model is the mouse, which shares 95% gene homology with humans [123]. While no animal will depict human physiology with 100% accuracy, model organisms provide us with valuable pre-clinical data for eventual study and application in humans. For example, the successful treatment of Type I diabetes by insulin is owed to the dog model that was used by Banting and McLeod, recipients of the Nobel Prize in 1921 [124]. However, although animal models may have contributed much to parturition research, there are some key differences to discuss between human and animal mechanisms of regulation in relation to the endocrine system that can influence the cross-species

interpretation of research findings. A summary of key differences between human and mouse parturition is provided in Table 1.

1.2.1. Progesterone Synthesis and Function. Key differences in the hormonal control of parturition between mammalian species include progesterone synthesis and regulation. During ovulation, the mature ovum is released into the fallopian tube, leaving behind a fluid filled sac called the ovarian follicle. This follicle then develops into the corpus luteum, the primary source of progesterone during the first seven weeks of pregnancy in humans [125]. During this period, progesterone suppresses the secretion of gonadotrophins follicle stimulating hormone and luteinizing hormone to prevent another ovulation. In the rabbit, rat, and mouse, the corpus luteum is the primary source of progesterone throughout gestation, whereas in the sheep, guinea pig, and human, the placenta eventually takes over as the dominant source of progesterone [125].

Progesterone maintains uterine quiescence by decreasing myometrial contractility, inhibiting myometrial gap junction formation, downregulating the synthesis of PG and of oxytocin (OT) receptors, and suppressing the opening of calcium channels in the myometrium [125, 126]. Progesterone also increases the expression of TIMP metallopeptidase inhibitor 1 in the cervix, which subsequently inhibits collagenolysis and cervical softening [127]. As such, it was proposed initially by Arpad Csapo that a ‘progesterone block’ regulates a decrease in progesterone that leads to the end of uterine quiescence [128]. It has since been confirmed that labour follows the systemic withdrawal of progesterone in rats, mice, sheep, and rabbits [129, 130] (Fig. 4). In contrast, systemic progesterone levels rise towards the end of pregnancy in humans and guinea pigs.

Table 1. Key Differences between Human and Mouse Parturition

	Human	Mouse
Primary Source of Progesterone	- First seven weeks: corpus luteum - After seven weeks: placenta	- Corpus luteum
Trends in Systemic Progesterone	- Rises towards the end of gestation	- Falls starting at around gestational day 16.5
Maternal Control of Labour	- Functional withdrawal of progesterone occurs, leading to the end of uterine quiescence	- COX-1 induces $PGF_{2\alpha}$ synthesis - $PGF_{2\alpha}$ induces luteolysis - Luteolysis results in the systemic withdrawal of progesterone - Progesterone withdrawal leads to the end of uterine quiescence
Chemokines	- IL-8	- CXCL1/KC - CXCL2/MIP-2 - CXCL5-6/LIX
Inducible Form of Cyclooxygenase	- COX-2	- COX-1
Fetal Control of Labour	- No evidence for one	- SP-A

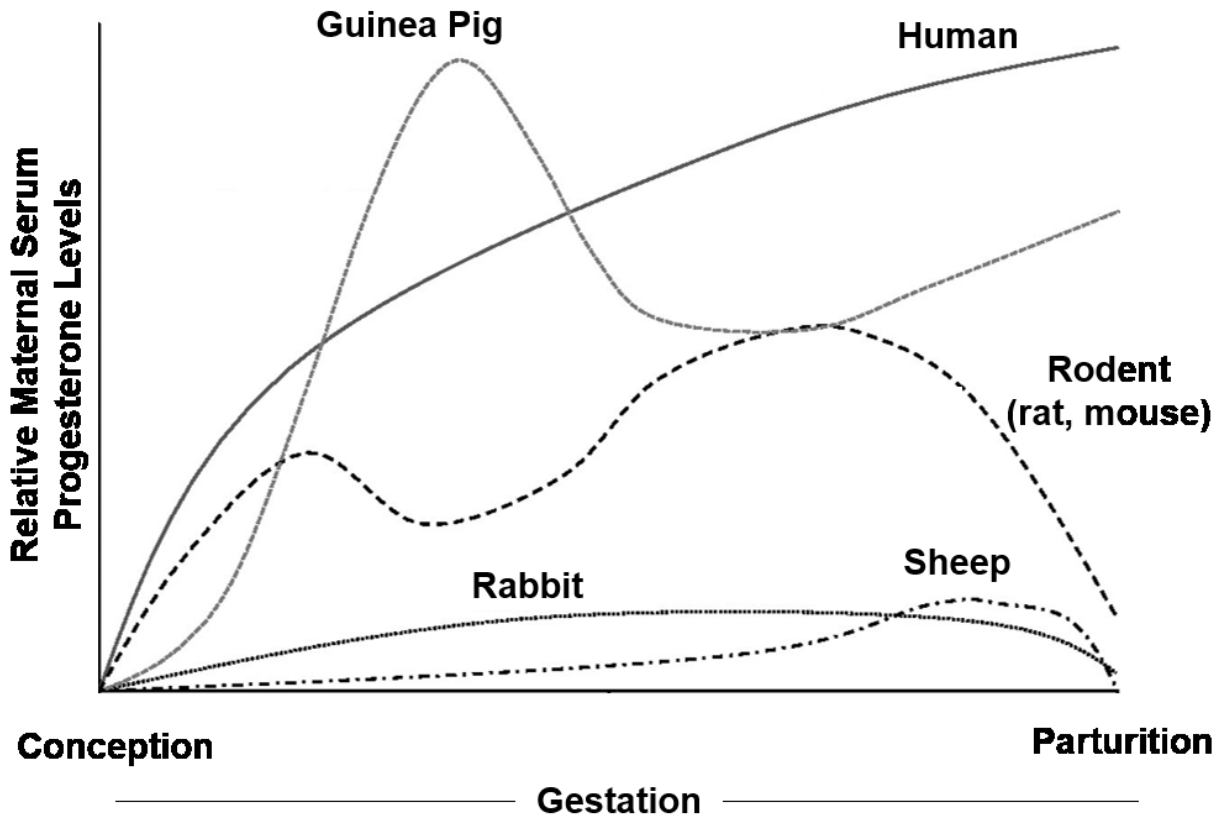

Figure 4. Relative maternal serum levels of progesterone throughout gestation in various animal species, adapted from Mitchell and Taggart, 2009 [57].

1.2.2. Progesterone Withdrawal as a Regulatory Mechanism of Parturition. In animals that depend on the corpus luteum as the primary source of progesterone, it has been suggested that the timing of parturition is regulated by $PGF_{2\alpha}$ [131]. An increase in $PGF_{2\alpha}$ towards the end of pregnancy mediates luteolysis, or degradation of the corpus luteum, resulting in the systemic withdrawal of progesterone. This has been evidenced by mice knockouts for the $PGF_{2\alpha}$ receptor gene that cannot deliver; in these mice, $PGF_{2\alpha}$ cannot initiate luteolysis and cause a systemic withdrawal of progesterone [132]. Moreover, the normal timing of birth in these mice can be restored by removing the corpus luteum physically via ovariectomy [132]. Further evidence that progesterone withdrawal is the cause of labour onset is that blocking progesterone activity induces labour in rats and mice [133, 134].

In sheep, the timing of parturition is also controlled by progesterone withdrawal. However, unlike rats, mice and rabbits, the dominant source of progesterone towards the end of gestation is the placenta instead of the corpus luteum, and the initial stimulus that triggers progesterone withdrawal is fetal cortisol [135]. The maturing fetal hypothalamus secretes corticotropin-releasing hormone (CRH), stimulating the pituitary to produce adrenocorticotropin-releasing hormone (ACTH), which induces the fetal adrenal gland to produce cortisol [136]. Cortisol then stimulates the sheep placenta to synthesize 17-α-hydroxylase cytochrome P450, an enzyme that catalyzes the conversion of progesterone to estrogen [137]. This process leads to a systemic withdrawal of progesterone and an increase in systemic estrogen.

In the uterus, estrogen promotes the expression of UAPs including PGE_2, $PGF_{2\alpha}$, PG receptors, OT receptors, connexins, α-adrenergic agonists, MLCK, and calmodulin [138-141]. PGs stimulate MMP activity, which can degrade the ECM that holds the fetal membrane together or promote cervical ripening through a similar mechanism [17, 19]. PGs also stimulate

myometrial contraction directly, as does OT [21]. The primary gap junction protein, connexin-43 (Cx-43), allows for rapid transduction of the contractile signal, and α-adrenergic agonists stimulate muscle contraction through the specific binding of α-adrenergic receptors [142]. Both MLCK and calmodulin are important to muscle contraction: once calcium enters the cell, it binds to calmodulin and activates MLCK, which phosphorylates myosin light chain at serine residue 19 [142]. This event enables the myosin head to bind the actin filament, initiating muscle contraction. Therefore, the cortisol-mediated shift from progesterone to estrogen dominance in sheep promotes labour. This is further evidenced by the ability to induce labour in sheep by stimulating the hypothalamic-pituitary-adrenal axis via exogenous administration of CRH, ACTH, or cortisol, as well as by mimicking progesterone withdrawal with RU-486, a competitive progesterone receptor antagonist [143, 144].

In contrast to rat, mouse, rabbit, and sheep parturition, human parturition is not dependent on a systemic withdrawal of progesterone, and administration of glucocorticoids has no effect on the timing of labour [145]. The primary source of progesterone in humans during late gestation is the placenta instead of the corpus luteum, and therefore $PGF_{2\alpha}$-mediated luteolysis does not lead to the same decrease in systemic progesterone that it does in rats, mice, and rabbits. Furthermore, fetal glucocorticoids in humans does not induce 17-α-hydroxylase cytochrome P450 in the placenta as it does in sheep, and therefore does not result in the conversion of progesterone to estrogen. This discrepancy originates from the differences in steroidogenesis between the sheep and human: while progesterone is converted to estrogen in the sheep, human estrogen is formed from androstane skeleton (C_{19}) precursors supplied by the adrenal gland [146].

In humans, the current theory is that instead of a systemic withdrawal of progesterone, there is a ‘functional withdrawal’ of progesterone by the modulation of progesterone activity

[147]. This functional withdrawal could be achieved by 1) regulating the expression of nuclear progesterone receptor (nPR) isoforms, 2) decreasing interactions between nPRs and their target gene promoters, 3) regulating the expression of coregulators that interact with nPRs, and 4) increasing the expression of an endogenous nPR antagonist [148]. There are two nPR isoforms, progesterone receptor (PR)-A and PR-B, both of which have separate cellular and genomic actions [148]. With advancing labour a shift from low PR-A:PR-B ratio (~0.5) to a high PR-A:PR-B ratio (~3) occurs, resulting in increased expression of COX-2, IL-8, and IL-1β [149].

COX is interesting in that it is common in humans and other animals, but the functional isoforms differ between species. As described previously, the systemic withdrawal of progesterone in rats, mice, and rabbits is regulated by $PGF_{2\alpha}$-mediated luteolysis. This event is initiated by an increase in COX-1 in the uterine decidua and the luminal epithelium of the non-decidualized uterine endometrium during late gestation [131, 150]. This mechanism has been evidenced by pregnant *COX-1* knockout mice that exhibit impaired luteolysis and delayed parturition, with no alteration to the timing of embryo implantation or post implantation growth [84]. Furthermore, induction of progesterone withdrawal via ovariectomy or administration of RU-486 dampens the effect of *COX-1* knockout on delaying parturition [84]. In contrast, COX-1 is constitutively expressed in most human tissues; instead, COX-2 is the inducible form of COX in humans. The human *cox2* promoter is controlled by a multitude of regulatory transcription factors including NF-κB, cAMP response element-binding protein, specificity protein 1, AP-2, NF-IL-6, transcription elongation factor, E26 transformation-specific domain transcription factor and ccaat-enhancer-binding protein [151-153]. COX-2 expression increases in the human myometrium and fetal membranes during late gestation, resulting in an upregulation of PG synthesis that helps transition the uterus [154-160].

1.3. Other Control Mechanisms of Parturition. Outside immune and hormonal control, there have been other suggestions for the control of parturition including the secretion of fetal surfactant in mice. Furthermore, I will provide a speculative discussion as to how the differences between reproductive anatomy may have created a need for better regulation for the timing of parturition in humans over other species.

1.3.1. Fetal Surfactant. In mice, it has been suggested that there is a fetal component to the regulation of parturition in surfactant protein A (SP-A). SP-A is secreted from the murine fetal lung and can be detected in the amniotic fluid at GD17, rising progressively to spontaneous labour at GD19 [146]. Intraamniotic injections of SP-A results in preterm delivery within 24 hours as well as the migration of macrophages into the injected horn of the uterus [146]. Furthermore, injection of SP-A antibodies delays pregnancy by more than 24 hours [146]. The mechanism of action of SP-A is not fully understood, but SP-A is known to regulate the production of inflammatory mediators such as TNF-α, IL-1β, ROS, and NO metabolites that may contribute to labour onset [161]. In contrast to the mouse, the human amniotic fluid concentration of SP-A decreases prior to spontaneous labour, from 5.6 μg/mL to 2.7 μg/mL [161]. It has been suggested that this decrease in human amniotic SP-A is due to uptake by macrophages, degradation by proteases during labour, and incorporation into tubular myelin in preparation for neonatal exposure to environmental hazards immediately following birth [161]. This is yet another example of the differences between species in how they regulate parturition.

1.3.2. Reproductive Anatomy. One argument for why these differences exist is because of the variations in reproductive anatomy which result in more challenging labour for humans than other animals. Natural selection has given way to a smaller pelvis to more efficiently transmit force from the hind legs to the spine but has also resulted in a smaller passageway for the fetus [162]. The narrow birth canal is further challenged by our large head diameter [162]. There is very little room for the human baby in the maternal cervix, meaning that an overdue baby can be too large for vaginal birth. Therefore, the timing of parturition is likely to be more closely regulated in humans than other animals.

1.4. Preterm Birth. Our knowledge of parturition and the mechanisms that regulate it are important not only for our general knowledge, but also for understanding how labor might be regulated in a condition like preterm birth when a fetus is delivered before term at 37 weeks of gestation, when the fetus is still immature and may not be ready for the world outside the uterus. Preterm birth affects over 15 million babies annually, and preterm birth complications are the leading cause of death in children under the age of 5 [163]. Acute and long-term complications impact the respiratory, gastrointestinal, cardiovascular, hematologic, central nervous, and ocular systems of preterm infants, and lead to a high healthcare burden.

1.4.1. Complications of Preterm Birth. Respiratory issues can arise from pulmonary hypoplasia and low surfactant production, both of which are common in extremely preterm infants, and lead to inadequate oxygen uptake [164]. Furthermore, 80% of infants before 27 weeks of gestation develop respiratory distress syndrome (RDS), and some of them develop bronchopulmonary dysplasia (BPD), a condition that is associated with growth and neurological problems during

childhood [164]. BPD often has residual effects on pulmonary function later in life. The risk of developing BPD is exacerbated by high oxygen levels and airway pressures provided by mechanical ventilators [165].

Challenges to the gastrointestinal (GI) system include an undeveloped suck-and-swallow reflex that can reduce nutritional intake [164]. An undeveloped GI tract could also make it difficult to digest any ingested food. Respiratory problems can further agitate the GI system and cause necrotizing enterocolitis (NEC) in a preterm infant's already fragile intestinal lining, resulting in perforation of the wall, and spillage of the intestinal contents into the abdomen [164].

Preterm infants are also at higher risk of being born with an open ductus arteriosis, increasing their risk for intraventricular hemorrhage, NEC, BPD, and death [166]. They are also at greater risk for hypotension, apnea, and bradycardia. A study of >2.6 million children and young adults reported that PTB was associated with a high risk of heart failure, and that this risk was even greater in extremely preterm infants (<27 weeks of gestation) [167].

The hematologic system of preterm infants is threatened by iatrogenic intervention. Frequent blood sampling of preterm infants who have suppressed hematopoiesis and a shorter erythrocyte turnover rate may result in anemia of prematurity [164].

Another system that is sensitive to injury because of PTB is the central nervous system. Hypoxia, poor blood flow, and hypotension can culminate in brain injury. The white matter surrounding the neural ventricles are especially susceptible to injury in preterm infants [168]. Preterm infants are also at higher risk of bleeding in the brain.

Finally, the ocular system is at risk in preterm infants. Retinopathy of prematurity, a condition that can cause irreparable blindness in preterm infants, is heavily correlated with shortened gestational length [169].

Those who survive prematurity are more susceptible to social and health problems later in life. In 2008, 903402 infants born alive and without congenital abnormalities were studied, of which ~45000 were born preterm [170]. In this study, decreasing gestational age was associated with a higher incidence of medical and social disabilities in adulthood, as defined as having cerebral palsy, having mental retardation, or receiving a disability pension. Conversely, increasing gestational age was associated with a higher educational level, higher income, social security benefits, and the establishment of family.

Some of the long term physiological consequences of PTB include the increased risk for respiratory illness in later life, lower respiratory flow rates, and reduced diffusing capacity [171-173]. These effects are exacerbated by higher rates of BPD or RDS [174]. The survivors of prematurity are also at greater risk for metabolic and chronic renal disease [175, 176]. This is unsurprising as 60% of nephrons are formed late in gestation, and low nephron number at birth has been associated with the development of chronic renal and cardiovascular disease [177]. Key influences that could impair nephron development or cause renal damage during a preterm infant's early life include inflammatory cytokines, reactive oxygen species (ROS), and anti-angiogenic factors, all of which are associated with preterm birth [178].

These complications contribute to $5.8 billion of the $10.2 billion annual cost of neonatal care in the USA [179]. The estimated cost (in USD) per surviving baby born at 25 weeks of gestation is $202,700, and $46,400 for a baby born at 30 weeks of gestation [180]. Moreover, the estimated societal economic burden associated with PTB in 2005 was at least $26.2 billion [180].

Mothers of preterm infants are also at greater risk of psychological distress than mothers of full-term infants, and it has been reported that 40% of mothers of preterm infants demonstrate significant depressive symptoms on the Edinburgh Postpartum Depression Scale one-month

post-partum [181]. This is much higher than the national average of 10% of mothers in the United States [182].

Ultimately, both the medical and social consequences of PTB demand a solution, but there are currently no effective ways to treat PTB. This is in part due to the many different etiologies for PTB that make it hard to identify molecular targets for PTB intervention.

1.4.2. Etiology of Preterm Birth. Bacterial infection, maternal risk factors, genetic predisposition, gestational complications, and fetal control of labour are some of the many contributors to PTB.

Historically, bacterial infection has been thought to be the primary cause of PTB. Pathways of bacterial entry include ascension from the vagina through the cervix, hematologic dissemination through the placenta, iatrogenic introduction, and retrograde spread through the fallopian tubes [55]. Once these microorganisms reach their destination, they are recognized by pathogen recognition receptors and stimulate the production and release of PGs, inflammatory mediators, and MMPs, resulting in uterine contraction and fetal membrane degradation [55]. Previously reported microorganisms in the amniotic cavity include *Ureaplasma parvum* and *Ureaplasma urealyticum*, but the amniotic cavity itself is usually sterile for bacteria, and even if bacteria is present in the chorioamnion, it is not enough to trigger PTB alone [183]. Moreover, approximately 60% of human patients suffering from PTB with evidence of intraamniotic inflammation display no clinical signs of infection, and therefore sterile PTB is far more prevalent [184].

Several risk factors have been identified for PTB. Close temporal proximity to a previous pregnancy confers a 2x increased risk of PTB, and women with previous preterm deliveries have a 2.5x increased risk of PTB in their next pregnancy [185, 186]. Low BMI, low blood volume,

and a lack of blood flow are also associated with an increased risk of PTB [187]. Furthermore, a large body of evidence demonstrates that women experiencing high levels of psychosocial stress during pregnancy are at higher risk for preterm delivery [188-190].

Another suggestion as to why some women are more susceptible to PTB than others is genetic predisposition. Recent evidence points to DNA variants (CARD6, CARD8, DEFB1, FUT2, MBL2, NLP10, NLRP12, NOD2) that could predispose a pregnancy to PTB [191]. Moreover, multiple genetic mutations for proteins involved in dampening the immune response have been identified in women who delivered preterm.

In addition to genetic factors, a plethora of gestational complications increase the risk for PTB, including multiple pregnancies, preeclampsia, and preterm premature rupture of membranes (PPROM). PTB in multiple pregnancies is seven times more prevalent than it is in singleton pregnancies [192]. As for preeclampsia, the only existing cure is delivery, and immediate delivery is recommended if the preeclampsia is severe [193]. Both multiple pregnancies and preeclampsia are associated with low birth weight, which is associated with PTB [194]. Moreover, PPROM is responsible for an estimated third of preterm deliveries [195]. The pathophysiology of PPROM is multifactorial, and includes choriodecidual infection, low socioeconomic status, cigarette smoking, sexually transmitted disease, multiple gestation, and amniocentesis [195]. Because of all these different etiologic considerations, it has been difficult to develop a PTB therapeutic.

1.4.3. PTB Therapeutics. Previously tested therapeutics for PTB include tocolysis and cervical cerclage, which aim to delay the physical expulsion of the fetus. More recently, the focus of PTB therapeutic research has shifted to progesterone, because progesterone withdrawal has been

linked to the end of uterine quiescence. Unfortunately, there is little evidence that any these approaches have improved perinatal outcomes.

Tocolysis aims to delay preterm delivery so that alternative rescue treatments can be used to improve fetal health while prolonging gestation. For example, the application of corticosteroids can help mature the fetal lungs during this delay period. Tocolytic drugs can be subdivided into selective β2-agonists, nitric oxide (NO), cyclooxygenase (COX) inhibitors, oxytocin receptor (OTR) antagonists, and voltage-gated calcium channel (VGCC) inhibitors [196].

Selective β2-agonists stimulate the production of intracellular cAMP, which in turn decreases calcium stores and opens large potassium channels, hyperpolarizing the smooth muscle cells in the uterus [197]. It was reported that these drugs were more effective than placebo at delaying PTB for two days, but there was a reported lack of benefit for long-term tocolysis (>7 days), an enhanced maternal morbidity rate, and the onset of potentially fatal side effects including chest pain, cardiac arrhythmias, and pulmonary edema [197, 198]. Moreover, prolonged exposure to β-adrenergic receptor agonists can lead to negative side effects in the babies, including autism spectrum disorders, psychiatric disorders, poor cognitive and motor function, and changes in blood pressure [199].

COX inhibitors blocks PG synthesis. By doing so, it prevents PG-mediated uterine contractions brought about by increased intracellular calcium and enhanced conduction through myometrial gap junctions. Potentially fatal side effects of using COX inhibitors include premature fetal ductus arteriosis closure in the fetus, and gastric ulcer or asthma recurrence in the mother [200].

OTR antagonists function on the principle that the elevated levels of oxytocin during labour increases intracellular calcium levels, eliciting myometrial contractions. A prime example of an OTR antagonist is atosiban, which once claimed to be as effective as other drugs in delaying PTB with fewer maternal side effects than tocolytics [201]. However, atosiban lacks long-term efficacy (<48 hours) and there are not enough sufficiently randomized trials against placebo to affirm any significant outcome with atosiban.

VGCCs like nifedipine interfere with calcium ion transfer, promoting myometrial relaxation. The problem with nifedipine is that it negatively impacts maternal circulation. Furthermore, like atosiban, there are no sufficiently randomized trials against placebo to demonstrate a significant positive outcome associated with the use of nifedipine.

Overall, the evidence for the effectiveness of tocolytics on improving perinatal outcomes, or even delaying pregnancy at all, is controversial. It was found that there was a lack of randomized clinical trials for tocolytic drugs, many of which had an insufficient number of patients or did not compare their results to a placebo [202]. These limitations suggest that tocolysis is not a viable PTB therapeutic. At the very least, tocolysis does not significantly reduce neonatal respiratory distress and mortality rates [196].

Another method employed by physicians to delay PTB is cerclage, or cervical suturing. This method aims to close the cervix and keep the baby inside the womb until the pregnancy reaches term, and has generally been used in cases where a woman's cervix is incompetent and opens too early. A meta-analysis of 15 randomized trials of cerclage in singleton pregnancies found no evidence that cerclage had negative effects on the mother following birth [203]. More importantly, pregnant women were less likely to have PTB for births compared to controls

before 37, 34, and 28 weeks of gestation [203]. However, cerclage has been reported to have no effect on perinatal morbidity or mortality outcomes [203].

A major focus of PTB therapeutic research for the past decade has been on progesterone. The role of progesterone in parturition is complex. Its effects on the myometrium include 1) suppression of estrogen activity by inhibiting the replacement of estrogen receptors, and 2) depolarization of the uterus by directly binding progesterone receptors [204]. The ability of progesterone to maintain uterine quiescence has been questioned in humans because maternal and fetal concentrations of progesterone are sustained before labour onset [205]. In the context of PTB, subsets of women who have had a preterm delivery have also reported a progesterone deficiency [206]. Several randomized trials have been conducted to determine whether administration of progesterone can help maintain uterine quiescence and prevent PTB. The first randomized trial reported that patients who had received vaginal progesterone had lower rates of PTB than placebo, but that the rate of adverse events was not significantly different [207]. The second randomized trial, however, provided evidence of RDS reduction with vaginal application of progesterone and none for other adverse outcomes [208]. An individual patient meta-analysis of five studies suggested that vaginal progesterone was effective at preventing PTB <33 weeks as well as composite neonatal mortality and morbidity [209]. Since then, caproate was added to progesterone to prolong the life of the molecule, resulting in the compound 17α-Hydroxyprogesterone Caproate [210].

However, conflicting evidence arose regarding the efficacy of progesterone on delaying pregnancy. Therefore, the most comprehensive, largest randomized controlled trial to date was conducted to settle this controversy in a study called the OPPTIMUM study [211]. In this study,

it was determined that there is no difference in gestational age at delivery between those that received vaginal progesterone or placebo.

Ultimately, none of the existing therapeutics have been effective at preventing PTB or improving neonatal outcomes.

1.5. Rationale. It is evident from the discussions in this chapter that the timing of parturition is key to successful reproduction, and that there are many mechanisms that regulate it including immunological and hormonal systems of control. As described previously, the hormonal control of parturition in humans is much less understood than in rodents and sheep and is likely linked to functional progesterone withdrawal. In addition to the fact that progesterone withdrawal signals the end of uterine quiescence, the shift in the progesterone nuclear receptor isoforms that mediate this functional withdrawal have also been demonstrated to initiate the production and secretion of inflammatory cytokines, including IL-1β, bridging the hormonal control of parturition in humans to the immunological control of parturition. In this way, the current model for the regulation of parturition is illustrated in Fig. 5. However, as shown in this figure, there are gaps in our understanding with regards to what events regulate the leukocyte invasion phenomenon that precedes and happens parallel to the key pro-labor events that culminate in fetal expulsion.

Therefore, the main objective of this study is to better understand the ways in which leukocyte invasion of the gestational tissues is achieved during parturition and preterm birth. The importance of studying leukocyte invasion is multifold: 1) leukocyte invasion is a parturition phenomenon, and it may help us develop a diagnostic that can predict when birth will occur, term or preterm; 2) understanding how the body regulates the timing of leukocyte invasion could be insightful for manipulating the timing of parturition; 3) leukocyte infiltration

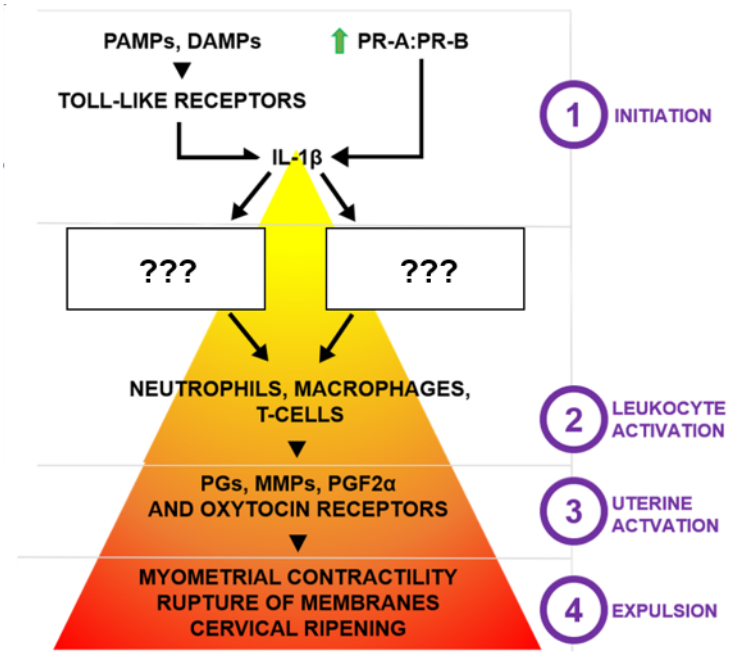

Figure 5. Current model for the regulation of parturition. Key pro-labor events that precede fetal expulsion include an increase in myometrial contractility, rupture of the fetal membranes, and ripening of the maternal cervix. Preceding these key events is the production of uterine activation proteins (UAP) in the gestational tissues including prostaglandins (PG), matrix metalloproteases (MMP) and oxytocin receptors. Before UAP production begins, leukocytes invasion occurs: leukocytes are recruited to the gestational tissues where they anchor themselves and release an array of cytokines that act as UAPs or stimulate the release of UAPs from the surrounding tissues. The mechanisms that stimulate leukocyte invasion are not well understood. It is thought that pathogen- and damage-associated molecular patterns (PAMPs, DAMPs) act on toll-like receptors to assemble an inflammasome and stimulate the production of IL-1β, a major cytokine that has been suggested to play a key regulatory role in parturition. A functional withdrawal of progesterone mediated by an increase in the progesterone nuclear receptor isoform (PR)-A:PR-B ratio can also stimulate the production of IL-1β.

of fetal organs is associated with fetal inflammation, and a better understanding of leukocyte invasion could lead to the development of therapeutics for these diseases; and 4) knowing how gestational tissues and leukocytes communicate may improve how we deliver future therapeutics.

These considerations will be tackled with three studies which will be described in Chapter 2, 3, and 4. Here I will describe each of the studies briefly. 1) Chapter 2 will focus on improving the Leukocyte Migration Assay (LMA), an experimental methodology that not only allows us to rapidly characterize leukocyte chemotactic activity and chemotactic factor activity, but also to harnessing the knowledge provided by leukocyte invasion as a parturition event to predict when labor will occur; this predictive test was developed by our lab and published in 2017 [4]. I will focus specifically on improving the speed, cost and precision of the assay. 2) The experiments in Chapter 3, using the improved LMA, will focus on understanding the mechanisms that regulate the leukocyte invasion phenomenon of parturition, with particular interest in the changes to the potency of chemotactic factors at the gestational tissues as well as the ability of peripheral leukocytes to respond to these factors in a mouse model of preterm birth. We will then take a brief look at where in the body the leukocytes may be being enhanced to migrate towards chemotactic factors. 3) Chapter 4 will look at the interactions of leukocytes and the fetal membranes to understand where the gestational chemotactic factors may be being secreted and how these may be modulated by IL-1β.

1.5.1. Chapter 2 Aims and Objectives. The aim of this study is to bring the LMA to a state where it is feasible enough for pre-clinical testing by improving the speed, cost and precision of the assay. Options will be explored to improve the leukocyte isolation step, chemotaxis step, and

quantification step. The various methods will be compared in terms of their advantages and limitations, and a direct material cost analysis will be performed. Using these methods, we will determine the best LMA protocol. In addition, a clinical implementation strategy will be developed as a proposal and discussed, as well as plans for pre-clinical testing of the improved LMA.

1.5.2. Chapter 3 Aims and Objectives. The aim of this study is to understand the regulatory mechanisms of leukocyte invasion better. To achieve this, we will first confirm the mechanism that has been demonstrated in other animal models, an increase in gestational chemotactic factors (GCF) as the pregnant animal approaches the estimated time of labor. Then, we will re-establish a mouse model of PTB that was developed previously by a collaborator and described in their publication [40]. We will confirm the model characteristics, determine whether leukocyte invasion occurs in this model of PTB, and characterize whether an association exists between leukocyte invasion and/or 1) an increase of GCF in the gestational tissues exists as well as 2) the phenomenon of enhanced peripheral leukocyte chemotaxis. We will also study whether PTB-induction by IL-1β is also associated with infiltration of leukocytes in the fetal brain and lung, two tissues that have been demonstrated to have higher mRNA abundance for proinflammatory cytokines in this mouse model [212]. In addition, we will study 101.10 (aka. Rytvela) in its capacity to prevent the effects of IL-1β administration in both the mother and the fetuses. Finally, we will take a brief look at whether non-enhanced leukocytes can be enhanced by maternal serum factors to help us understand where the enhancing effect is taking place.

1.5.3. Chapter 4 Aims and Objectives. In this study, we aim to look at the interactions between leukocytes and the fetal membranes in humans. Previous work from a now-graduated PhD student in our lab has demonstrated that merely bathing primary human myometrial and fetal membrane tissues together in the same media for 6 hours results in a multi-fold change in the mRNA abundance for proinflammatory cytokines in either tissue (Leimert and Olson, unpublished data). In a similar way, the gestational tissues and the invading leukocytes may communicate as it has been demonstrated in inflammatory conditions: for example, endothelial cells have been demonstrated to provide critical signals like macrophage colony-stimulating factor 1 precursor (Csf1) for the selective growth and differentiation of macrophages, which become capable of accelerating angiogenesis, promoting tumor growth, and adhere tightly with endothelial cells *in vivo* [213].

With relation to leukocyte invasion, the fetal membranes are a major site for leukocyte invasion during parturition in humans [4], and crosstalk between the leukocytes and the fetal membranes may further promote leukocyte invasion and inflammation of the gestational tissues. IL-1β in the fetal membranes has been immunolocalized to the leukocytes, and many other cytokines including IL-8 and IL-6 have been immunolocalized to the leukocytes in myometrium and cervix suggesting that leukocytes are a major source of cytokines and chemokines in the fetal membranes [214]. It is likely that the fetal membranes can amplify this response because they are a major source of inflammatory cytokines as described in the previous paragraph, cytokines that can stimulate leukocyte activity in secreting these cytokines and chemokines.

Therefore, the main objective of this study is to first understand whether fetal membrane secretions contain chemotactic factors. Then, we will determine whether these secretions are capable of stimulating leukocytes to secrete chemotactic factors, or to demonstrate enhanced

chemotactic response to a standard chemoattractant. Finally, we will determine whether these effects change when the fetal membranes are first pre-treated with IL-1β for 6 hours before their secretions are collected.

Chapter 2. The Leukocyte Migration Assay, a way to predict birth using maternal leukocytes.

2.1. Introduction. Preterm birth (PTB) is a birth that takes place before 37 weeks of gestation. Acute complications associated with PTB are the leading causes of death among children under five years of age [163]. An estimated 965,000 children have died due to PTB complications in 2013 alone, and this number continues to rise [163]. Moreover, the long-term motor, cognitive, visual, hearing, behavioral, health, and growth problems associated with PTB can bring about considerable emotional and economic burden. Despite this growing problem, there are no effective therapeutics for PTB as described in the previous chapter. In addition, even if a therapeutic was successfully developed, currently there are no means of diagnosing those at risk for PTB and determining to whom to administer the therapeutic.

2.1.1. Existing PTB Diagnostics. Several diagnostic methods for PTB have been explored, including assessment of maternal risk factors, sonographic measurement of cervical length, and detection of biochemical markers, but none have been able to improve birth outcomes.

Based on the maternal risk factors, physicians can attempt to narrow down who is at risk for PTB. Considerations include 1) demographic characteristics such as low socioeconomic status, poor antenatal care, advanced maternal age, or malnutrition; 2) behavioral characteristics such as smoking, illicit drug use, alcohol consumption, or laborious physical work; 3) obstetric history such as genetic predisposition, uterine malformation, or previous incidences of PTB, PPROM, cone biopsy, or cervical surgery; and 4) current pregnancy characteristics such as multiple fetuses, genital tract bleeding and/or infection, fetal malformation, PPROM, shortened

cervix, and other pregnancy complications such as preeclampsia or gestational diabetes [151, 152]. Unfortunately, overall risk factor assessment alone is unreliable, and fails to identify over 50% of pregnancies that deliver preterm [185, 215, 216].

Another diagnostic method that has been explored is the detection of a short cervix. Although a shortened cervical length may result from natural variation, it could be indicative of hemorrhage or infection leading to inflammation, uterine overdistention due to a condition like multiple fetuses, or subclinical contractions [217]. Short cervix as detected by transvaginal ultrasound in the midtrimester (18-24 wks) of pregnancy diagnoses a woman for the risk of PTB, but the reliability of this diagnostic is low due to limited ultrasound availability and operator expertise [217, 218]. Nevertheless, a cervical length of ≤15mm indicates that a patient has a 50% risk of PTB at <33 weeks of gestation [219]. Cervical length determination may be used in conjunction with other diagnostics to improve the accuracy of diagnosis.

Investigators have also looked to the detection of biochemical markers to predict PTB risk. This approach is advantageous for a rapid bedside diagnostic test because of its simplicity. However, it is becoming increasingly evident that single biomarker approaches are unlikely to be reliable enough for PTB diagnosis [220]. Previous attempts to identify biochemical markers for PTB diagnosis will be discussed as they have been discovered in the various biological fluids.

Amniocentesis has been used extensively to study the genome and proteome of the amniotic fluid, but the procedure risks eliciting PTB as well as causing fetal trauma and infection. Due to these risks, amniocentesis is a weak contender as a PTB diagnostic. Furthermore, despite the associations of PTB with several amniotic biomarkers such as IL-6, IL-8, IL16, IFNγ-inducible protein 10, annexin A2, CXCL11, secretory leukocyte peptidase

inhibitor, a disintegrin and metallopeptidase domain 8, sICAM1, and vICAM1, none of these in isolation have been predictive of PTB [159-164].

Alternatively, salivary markers have been proposed for PTB diagnosis. The main advantage of a maternal salivary biomarker over an amniotic fluid biomarker is the safety and ease of sample acquisition. Promising salivary biomarkers include salivary progesterone. A low concentration of salivary progesterone between 24-34 weeks of gestation has been reported in women at risk of early PTB (<34 weeks) [227]. A follow-up study has identified the cut-off value for predicting PTB before 34 weeks of gestation using salivary progesterone at 2575 pg/mL in women with at least one identifiable risk factor for PTB, with a sensitivity of 83%, specificity 86%, positive predictive value (PPV) 60%, and negative predictive value (NPV) 95% [228].

Like saliva, blood is easily accessible. However, blood is usually sampled from the systemic circulation, which may differ in biochemical composition from the local circulation near the gestational tissues. The chemical biomarkers of PTB in a blood sample may therefore be diluted amongst the thousands of other proteins in circulation. Despite this limitation, a few potential blood biomarkers for PTB have been identified. Plasma urocortin in women with symptoms of threatened PTB has been reported to be predictive of PTB with a sensitivity of 80%, specificity 100%, and PPV 100% within 7 days of sampling [229]. However, the usefulness of this diagnostic has been called into question because the women that were identified for PTB risk in the study were already presenting labour symptoms. Another attempt to identify a diagnostic biomarker in blood was the analysis of 27 proteins in women presenting threatened preterm labour. IL10, sIL-6R, LTA, CCL4, MMP9, BDNF, CSF2, and sTNFR1A were reported as being significantly differently expressed compared to control [230]. Each

individual biomarker, however, was less predictive of PTB than cervical length measurement [230]. One multivariable combined model using serum IL-10 and RANTES levels and cervical length was able to predict PTB within 7 days with sensitivity 74%, specificity 78%, PPV 76%, NPV 86%, and AUC 0.88 [230], but no follow-up studies have since been published.

Like saliva and blood, maternal cervicovaginal fluid (CVF) is safely and easily accessible. CVF is an amalgamation of secretions from the vagina, cervix, decidua, and fetal membranes. CVF therefore receives input from multiple gestational tissues and is likely effective for monitoring maternal and fetal health. Two biomarkers that have been studied extensively in CVF are fetal fibronectin (FFN) and phosphorylated insulin-like growth factor binding protein-1 (phIGFBP1).

FFN is a large glycoprotein that is secreted by the trophoblast. It provides structural support and adhesion of the fetal membranes to the uterus by binding to cell surface receptors, collagen, proteoglycans, and other fibronectin molecules [231]. It has been suggested that the presence of FFN in CVF signifies breakdown of the fetal membranes and the release of FFN and other matrix molecules into the vagina [232]. It has also been suggested that FFN actively participates in fetal membrane breakdown and labour induction by inducing MMP and PG synthesis [232]. FFN presentation in CVF has been used as a clinical marker for PTB previously, but this diagnostic is unreliable: in symptomatic patients, the PPV and NPV for predicting PTB within 7 days of FFN presentation are 13% and >99% respectively, and in asymptomatic patients, the PPV is 17-25% depending on the gestational age of the patient at testing [109, 233]. The FFN diagnostic is currently used to determine whether a symptomatic patient requires immediate transfer to a tertiary healthcare facility [232].

phIGFBP1, on the other hand, is secreted by decidual cells into the amniotic fluid [232]. The presence of phIGFBP1 in CVF indicates leakage from the amniotic fluid when the fetal membranes detach from the decidua. As with FFN, phIGFBP1 presentation as a predictive marker of PTB is unreliable because it lacks a suitable PPV in asymptomatic women [234].

2.1.2. Leukocyte Migration Assay. Recently, our lab has developed an assay to diagnose PTB within 7 days of labour onset [4]. Our patented bioassay, the Leukocyte Migration Assay (LMA), functions on the basic principle that leukocytes exhibit enhanced chemotaxis as a woman approaches spontaneous labour, whether term or preterm [4]. A schematic for the original LMA is shown in Fig. 6, and will be described in more detail in the methods section of this chapter. The LMA functions on the basic principal that leukocyte invasion of the gestational tissues precludes parturition.

One of the mechanisms that have been suggested to contribute to leukocyte invasion is an enhancement of leukocyte chemotaxis. CD11b+ neutrophils at term and preterm labour are more responsive towards a standard chemoattractant than are neutrophils at TNL or PTNL, and total leukocytes at TL are more responsive than those at TNL towards TL fetal membrane chemoattractants [4, 61]. In addition, enhanced leukocyte chemotaxis has been confirmed in rats [3]. The LMA translates this knowledge to predict the timing of labour. In brief, 100,000 leukocytes are loaded into the top wells of a modified Boyden chamber, and chemoattractants are loaded into the bottom wells. The top and bottom wells are separated by a porous polycarbonate filter that prevents unstimulated movement of leukocytes to the bottom wells, except when they are stimulated by chemoattractants. After incubation at 37^{o}C in 5% CO_2 for 90 minutes, the chemoattracted leukocytes are quantified using flow cytometry. A sufficiently high number of

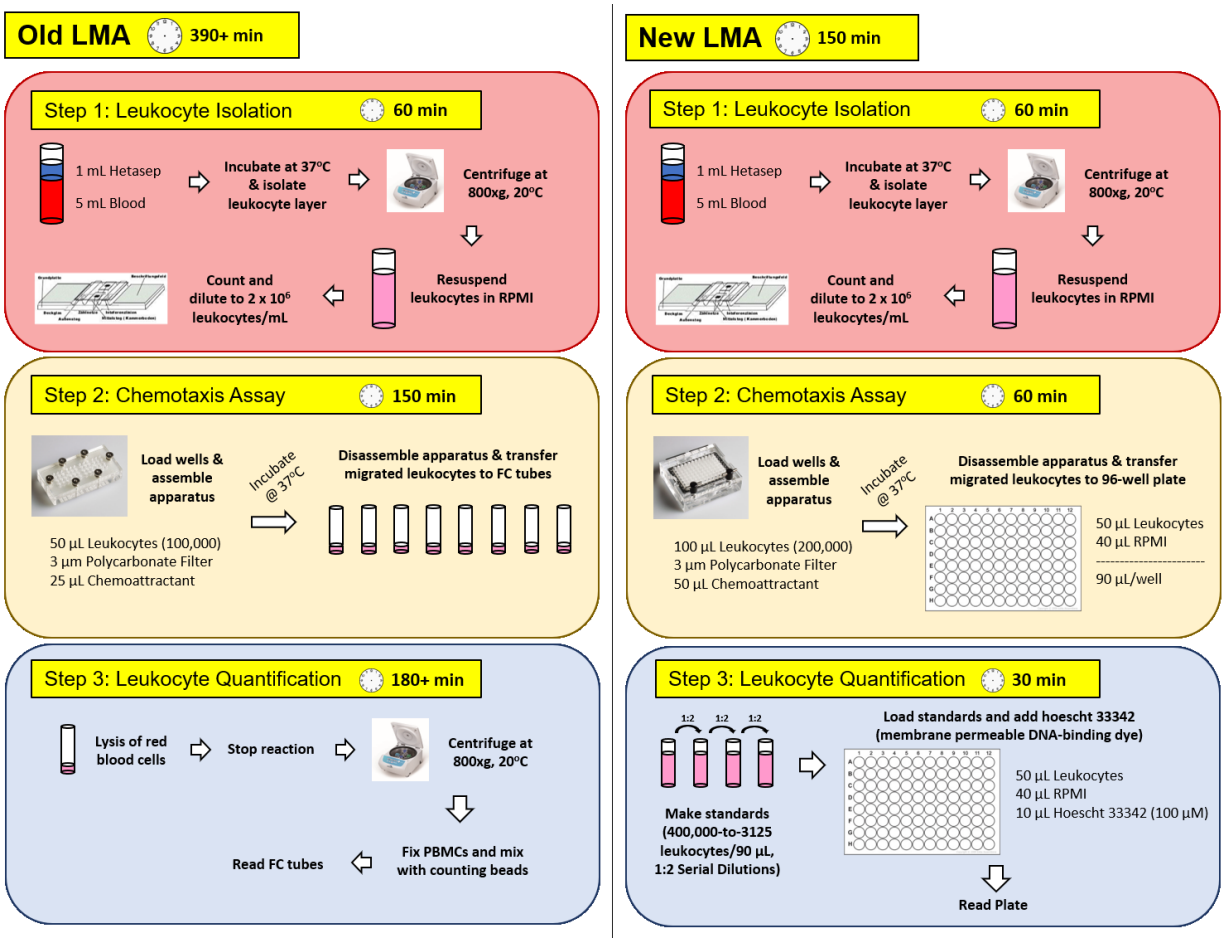

Figure 6. A Comparison of the Old and New Leukocyte Migration Assays for a Full Plate of Samples. Leukocytes were isolated from human blood using HetaSep, an erythrocyte sedimentation reagent. RPMI was used as the resuspension buffer for leukocytes. Leukocytes then underwent chemotaxis in either a 48-well or 96-well chamber at 37°C for 90 or 30 minutes respectively. Upon disassembly of the chemotaxis chamber, leukocytes were either transferred to flow cytometry tubes or to a 96-well clear bottom plate for quantification.

chemoattracted leukocytes translates to an enhancement of leukocyte chemotaxis that is predictive of parturition.

Receiver operating characteristic (ROC) curve parameters indicate that the chemoattraction of 37,082 total leukocytes out of 100,000 seeded in the upper wells of the chemotaxis chamber is predictive of labour within 7 days, with a sensitivity of 78.1%, specificity 88.9%, PPV 91.4%, NPV 72.7%, and area under the curve (AUC) of 0.83 [4]. These parameters are statistical measures of the performance of a binary classification test such as the LMA. With regards to the LMA, sensitivity refers to the probability that a positive result correctly identifies that a woman will undergo labour within 7 days. On the other hand, specificity refers to the probability that a negative result correctly identifies that a woman will not undergo labour within 7 days. In contrast, PPV is proportion of subjects with a positive test who go on to spontaneously deliver within 7 days, whereas the NPV is the proportion of subjects with a negative test who do not spontaneously deliver within 7 days. The AUC is a measure of a test's accuracy, and an AUC of 0.83 classifies the LMA as good clinical test.

A major limitation of this assay, however, is that the monetary and temporal cost of each assay could be prohibitive to its clinical implementation. The original LMA by Takeda *et al* [4] is more than six hours long at a cost of over $135 per assay plate (up to 24 samples in triplicate determination). Moreover, the assay is technically difficult to perform. There is a risk of puncturing the filter during disassembly of the chamber leading to sample contamination and a risk of losing sample while washing the chemoattracted leukocytes. In addition, the lower chamber is difficult to wash due to the small well diameter, which increases the risk of contamination of the bottom wells with chemoattractant or detergent. Finally, there is high

variable counting precision associated with flow cytometry due to the technical difficulty of sample preparation for an untrained operator.

2.1.3. Rationale, Objectives, and Experimental Approach. As demonstrated by early clinical evidence [2], the LMA has the potential to be the first effective diagnostic test for the prediction of PTB. During my Master's training, I had the opportunity to streamline the LMA protocol. My objectives were to make the LMA faster, cheaper, more accurate, and more reliable than the original assay. To accomplish these objectives, I identified three stages in the LMA for the possibility of improvement: leukocyte isolation, chemotaxis and quantification. For each of these stages, I looked at published methodologies in the literature that could accomplish the same task with better performance characteristics.

For isolating leukocytes, I compared the current system of erythrocyte sedimentation using HetaSep and a new system of magnetic-based negative selection of neutrophils using MACSxpress (Miltenyi Biotec, Bergisch Gladbach, Germany). In this new system, erythrocytes are also coagulated and sedimented, and non-target (non-neutrophil) cells can also be removed simultaneously by immunomagnetic depletion using antibody-coated magnetic beads. The first apparent issue might be that this technique is unsuitable because it only isolates neutrophils, but I thought that this might even be better for the assay; from the original publication where the LMA was first proposed, the greatest population of migrating leukocytes by far were the granulocytes [4], and most likely neutrophils given that most (>90%) of the circulating granulocytes are neutrophils. Therefore, the projected benefits of this technique were not only that it would take 20 minutes instead of 60 for separating our leukocytes, but also that our major population of migrating leukocytes would be our primary target group, the neutrophils. The projected

limitations of this assay was that we were not certain whether neutrophil chemotaxis would be affected by the absence of the other leukocytes, and that it would be much more expensive ($60 CAD for the new method vs $1 CAD for the old method per assay).

For the chemotaxis assay, I compared the 48-well chemotaxis chamber system, (Neuro Probe Inc., MD, USA) which was used in the original publication [4], to the 96-well chemotaxis chamber system (Neuro Probe Inc., MD, USA) and a modified under-agarose assay for measuring chemotaxis based on the system described by Nelson *et al* [235]. The projected advantages of the 96-well system were that it would offer a greater surface area for chemotaxis and thus would decrease the length of time needed to get significant leukocyte migration, and that the larger wells would be easier to clean thus improving the precision between experiments. The limitation of the 96-well system was that it was more expensive for the chamber apparatus (>$3000 for 96-well vs <$1000 for 48-well) and per filter ($15 per 96-well filter vs $1 per 48-well filter). The under-agarose assay, however, would be extremely cheap to make in a petri dish, and chemotaxis wells could be punched out using sterile, stainless-steel 2 mm-diameter tissue punches. The primary limitation for the under-agarose assay was how long it would take for significant migration to take place, many of them standardized to 2 hours for human peripheral neutrophils [236, 237].

To improve the quantification step to count migrated leukocytes, I initially explored a few different methods such as the addition of NucRed and NucBlue (Thermo Fisher Scientific Inc., Ottawa, ON, Canada), two DNA-binding, fluorescence-labelling reagents primarily used for fluorescence microscopy. Preliminary trials demonstrated that NucBlue was the better of the two with regards to standard curve characteristics, but NucBlue was an expensive option at $138 per 6 bottles, which realistically could only stain twelve full 96-well plates. Upon discovering that

NucBlue was basically an optimized solution of Hoechst 33342, a DNA-binding fluorescence dye, I sought to characterize fluorescence spectroscopy with Hoechst 33342 as a tool for rapidly quantifying neutrophils, as it was significantly cheaper at $90.50, which could stain over 2000 full 96-well plates. I optimized the concentration of Hoechst 33342 that would give a linear range over 0-200,000 total human leukocytes and compared its performance characteristics against flow cytometry with counting beads, which was the originally proposed method of quantifying leukocytes, and against counts with a hemocytometer. The projected advantages of using Hoechst 33342 were that it would be cheaper and faster. A limitation was that we wouldn't be able to quantify the various subpopulations of leukocytes, but that wasn't a huge concern considering that we only cared about the total leukocyte migration for clinical implementation as a PTB diagnostic.

2.2. Methods. *Experimental Groups.* Human whole blood was collected from TL pregnant women at the Royal Alexandra Hospital (Edmonton, AB, Canada). Labour was documented by cervical dilation of greater or equal to 4 cm in the presence of uterine contractions. Women with clinical infection, premature rupture of membranes, or dysfunctional labour, and recipients of PGs or artificial OT were excluded from the study. Women with diabetes mellitus, immunological problems, non-singleton pregnancies, intrauterine growth restriction, and preeclampsia were also excluded.

As the standard chemoattractants for these experiments, we used a pair of potent and non-potent chemoattractant for human total leukocytes. These were either human or mouse chemoattractants: 1) Intact placentas were collected with consent from TNL women undergoing an elective caesarean section or from TL women undergoing spontaneous labor (Royal

Alexandra Hospital, Edmonton, AB). Human fetal membranes were excised as described previously by Takeda *et al* [2] and normalized to wet tissue weight in Dulbecco's Modified Eagle's Medium (DMEM). Gestational chemotactic factors (GCF) were extracted via homogenization and centrifugation to get rid of the cellular debris. GCF were then pooled for the TNL FM (non-potent chemoattractant) and TL FM (potent chemoattractant) groups (n=6). 2) Pregnant mice were euthanized at GD15 and GD19 and their lower uteri (mLU) were collected and normalized to wet tissue weight in Dulbecco's Modified Eagle's Medium (DMEM). GCF were extracted from the lower uteri via homogenization and centrifugation to get rid of the cellular debris. GCF were then pooled for the GD15 mLU (non-potent chemoattractant) and GD19 mLU (potent chemoattractant) groups (n=6).

Original Leukocyte Migration Assay. Total leukocytes were isolated using HetaSep (Stemcell Technologies Canada Inc., Vancouver, BC, Canada) as specified by the manufacturer, and resuspended in Roswell Park Memorial Institute medium 1640 (RPMI 1640). Chemoattractants were loaded in triplicate determination into the lower wells of a 48-well chemotaxis chamber (Neuro Probe Inc., MD, USA). A polycarbonate filter (3 μm pores) was placed on top, and total leukocytes (100,000 cells) were loaded into the upper wells of the chamber. Leukocytes were left to undergo chemotaxis at 37ºC in 5% CO_2 for 30 minutes.

Chemoattracted leukocytes were then individually transferred from each well to BD Falcon™ 5 mL polystyrene round bottom tubes (Thermo Fisher Scientific Inc., Ottawa, ON, Canada) and treated with OptiLyse C (Beckman Coulter, Mississauga, ON, Canada) to lyse the red blood cells. The reaction was stopped and leukocytes were pelleted using centrifugation at 1,000 x *g* for 10 minutes at 4ºC. The supernatant was discarded, and the leukocytes were fixed

with 1% paraformaldehyde in phosphate buffered saline and mixed with a standard concentration of CountBright Absolute Counting Beads (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's recommendations. Samples were run on a flow cytometer (BD FACSCanto^{TM} II, BD Biosciences, San Jose, CA, USA) at the Flow Core facility at the Faculty of Medicine and Dentistry (University of Alberta, Edmonton, AB). Forward scatter and side scatter were plotted, the beads were gated, and acquisition stopped when 1,000 beads were recorded. Lymphocyte, macrophage, and neutrophil counts were gated and compared to the bead counts to determine the concentrations of each leukocyte subpopulation in solution. From these concentrations, the total number of leukocytes that migrated was calculated.

Alternate Leukocyte Isolation Method – MACSxpress. Neutrophils were isolated according to the manufacturer's protocol. In brief, an erythrocyte sedimentation reagent and antibody-coated magnetic beads are added to whole blood, mixed, and incubated for 15 minutes in a magnetic field that surrounds the tube. During this time, erythrocytes are sedimented, and non-neutrophils are pulled to the sides of the tube, leaving a neutrophil layer in the middle of the tube that can be isolated. Viable leukocytes were counted using trypan blue exclusion. Chemotactic activity was measured either prior to washing and resuspension in RPMI, or after.

Alternate Chemotaxis Assay Method – 96-Well Chemotaxis Chamber. Chemoattractants were loaded in triplicate determination into the lower wells of a 96-well chemotaxis chamber (Neuro Probe Inc., MD, USA). A polycarbonate filter (3 μm pores) was placed on top, and total leukocytes (200,000 cells) were loaded into the upper wells of the chamber. Leukocytes were left

to undergo chemotaxis at 37ºC in 5% CO_2 for 30 minutes. Chemoattracted leukocytes were transferred to a 96-well, black, clear bottom plate.

Alternate Chemotaxis Assay Method – Modified Under-Agarose Assay. This assay has been described previously [U, V, W] with minor modifications. In brief, agar plates were made in Falcon 35 mm x 10 mm culture dishes: each dish was filled with 3 mL of an 1.2% agarose solution containing 50% H_2CO_3-buffered hank's balanced salt solution, 50% RPMI-1640 culture media and 20% heat-inactivated fetal calf serum. After solidification of the agar, 2 mm sterile, stainless steel tissue punches were used to cut three sets of two wells 2.4 mm apart. Thin, sterile plastic separators were inserted between each set of wells to prevent cross-contamination of chemotactic factors. Gels were left to equilibrate for 1 hour at 37ºC in 5% CO_2. One well was then filled with chemotactic factor and the other well was filled with total leukocytes (100,000 cells). The area between the wells was checked using brightfield microscopy every 15 minutes to assess leukocyte migration. The number of leukocytes left in the original well were counted using flow cytometry and subtracted from the original number of seeded leukocytes (100,000) to determine how many leukocytes were chemoattracted out of the well.

Alternate Leukocyte Quantification Method – Fluorescence Spectroscopy. Serial dilutions of 2:1 were used to generate a standard curve of known leukocyte concentrations from the original patient, ranging from 400,000 to 3,125 cells. Hoechst 33342 (final concentration: 1 pM, excitation/mission: 350/361 nm) was added to reach a final volume of 100 µL per well. Fluorescence in each well was measured using a Fluoroskan Ascent fluorescence spectrophotometer (Thermo Fisher Scientific Inc., Ottawa, ON, Canada), and the number of

chemoattracted leukocytes was calculated using 4 parameter logistic regression, the mathematical model used commonly to determine concentrations of molecular compounds in enzyme-linked immunosorbent assays. This regression model is suitable for quantifying leukocytes because it is a model that has an eventual maximum and minimum y-axis value, just like in our system which can range from 0-200,000 leukocytes; this is more reasonable than a linear regression model which assumes that the measurements have no maximum nor minimum.

2.3. Results. A summary of findings for each of the proposed changes to the LMA is detailed in Table 2 and a direct material cost analysis is detailed in Table 3.

Leukocyte Isolation from Whole Blood – MACSxpress Neutrophil Isolation. The MACSxpress neutrophil isolation kit was able to isolate neutrophils from whole blood successfully. A preliminary assessment of neutrophil chemotactic function using an LMA demonstrated that the resulting unwashed neutrophils at the end of the isolation protocol as described by the manufacturer had 0% chemotactic activity in response to either GCF from TL or TNL FM (Fig. 7). On the other hand, washed neutrophils isolated via MACSxpress demonstrated a greater chemotactic response than total leukocytes isolated via HetaSep to GCF from both TL and TNL FM.

Chemotaxis Assay – 96-Well Chemotaxis Chamber. A time course was done for the 96-well system to determine the ideal incubation time for our chemoattractants (human TL & TNL FM GCF and mouse GD15 & GD19 LU GCF) and number of total leukocytes (200,000 cells per well). At 30 minutes of incubation at 37ºC in 5% CO_2, there was a non-maximal but significant

Table 2. Comparison of alternate procedures for the leukocyte migration assay.

	Old LMA (48 Wells)	**MACSxpress Neutrophil Isolation**	**96-Well Chemotaxis Chamber System**	**Modified Under-Agarose Assay**	**Fluorescence Spectroscopy**
Overview	Method for isolation of total leukocytes + chemotaxis assay + quantification of migrated leukocytes.	Method of isolating human neutrophils from whole blood.	Method of comparing chemotaxis of leukocyte to chemotactic factors.	Method of comparing chemotaxis of leukocyte to chemotactic factors.	Method of counting migrated leukocytes.
Length of Procedure	Leukocyte Isolation: 60 min Chemotaxis Assay: 150 min Quantification: 180+ min **Total**: 390+ min	Without the Washing & Resuspension step: 30 min With the Washing & Resuspension step: 60 min **Total**: 30/60 min	Assembly: 15 min Chemotaxis: 30 min Disassembly: 15 min **Total**: 60 min	Making Agar Plates: 30 min Equilibration: 60 min Chemotaxis: 120 min **Total**: 210 min	Transfer of migrated leukocytes to a clear-bottom 96-well plate and adding Hoechst 33342: 10 min Incubation of cells with Hoechst 33342: 20 min **Total**: 30 min
Total Cost*	$244.17 for 16 patients	$62.02 for 1 patient	$15.72 for up to 32 patients	$2.50 for 1 patient	$4.81 for up to 6 patients
Advantages		- Simple protocol - Pure neutrophils	- Rapid and inexpensive - Easy to handle - Easy to wash & reuse	- Inexpensive - Disposable	- Rapid and inexpensive - High precision
Limitations	- Risk of puncturing the filter and interexperimental contamination due to small size of wells - Can lose sample during washing of post-migration leukocytes for flow analysis - Flow cytometry has variable counting precision	- No migration was detected if neutrophils are not washed and resuspended in RPMI - Expensive	- Difficult to use efficiently in clinic because the filter is single-use; good for testing multiple patients at once, but not for patients that need to tested at different times (ie. walk-in patients)	- Leukocytes get trapped in the well edge - Agar often lifts slightly from the bottom of the culture plate, leading to spillage of well contents	

*Direct material cost analysis can be found on Table 3. Costs for the old LMA protocol was calculated for a full plate (48-wells)

Table 3. Direct material cost analysis of alternate procedures for the leukocyte migration assay.

	Old LMA (48 Wells)*		**MACSxpress Neutrophil Isolation**		**96-Well Chemotaxis Chamber System**		**Modified Under-Agarose Assay**		**Fluorescence Spectroscopy**	
General (Tubes, Tips)	Tubes Tips	$9.41 $10.82	Tubes Tips	$0.54 $0.18	Tubes Tips	$0.54 $0.18	Tubes Tips	$0.17 $0.30	Tubes Tips	$0.50 $0.26
Reagents	16 mL HetaSep 16 mL Optilyse C 800 µL Counting Beads	$16.16 $76.96 $60.00	Isolation Kit	$60.00			Agarose HBSS**	$0.10 $0.25	Hoechst 33342 (1 pM)	$0.05
Other Consumables	1x Filter (3 µm pores) 150 mL RPMI-1640 16x Vacutainer (heparin-coated)	$2.80 $9.75 $8.27	RPMI-1640	$1.30	1x Framed Filter (3 µm pores)	$15.00	Culture Plate RPMI-1640 FCS***	$0.62 $0.07 $0.99	Clear Bottom 96-Well Plate	$4.00
Other Costs	Flow Cytometer Operational Cost	$50.00								
Total Cost	$244.17 for 16 patients		$62.02 for 1 patient		$15.72 for up to 32 patients		$2.50 for 1 patient		$4.81 for up to 6 patients	

*Costs for the old LMA protocol was calculated for a full plate (48-wells) with 16 patient samples (3-wells per patient)
**50% H_2CO_3-Buffered Hank's Balanced Salt Solution
***20% Heat-Inactivated Fetal Calf Serum

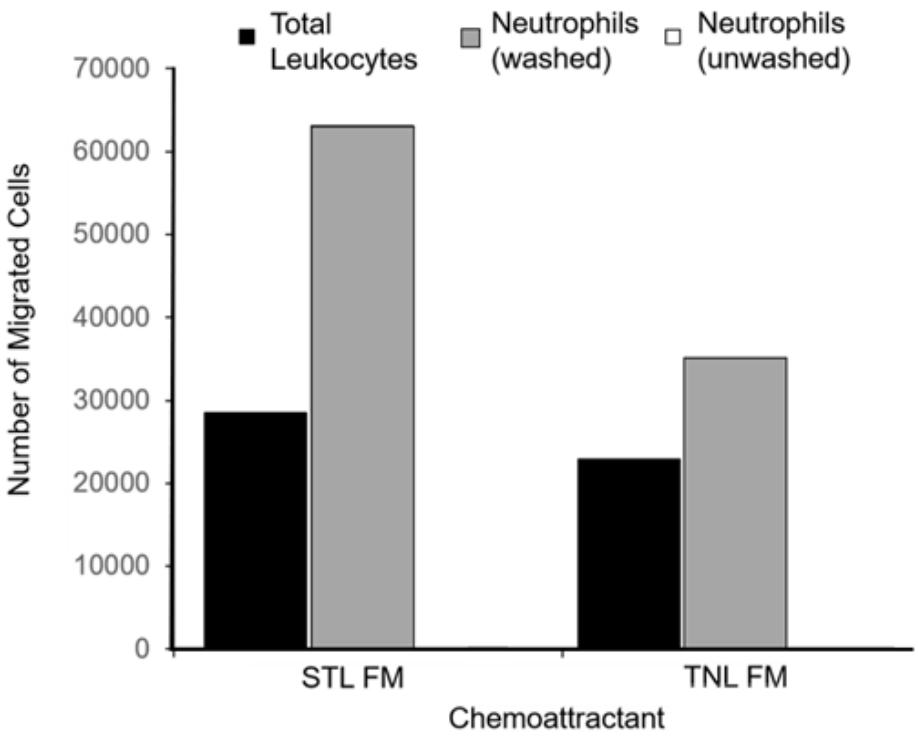

Figure 7. Comparison of various leukocyte or neutrophil isolation methods (n=1 each). Total leukocytes or purified neutrophils were isolated using HetaSep or MACSxpress neutrophil isolation kit as directed by the manufacturer respectively. The neutrophils were either left alone following the protocol, or washed in PBS, centrifuged, and resuspended in RPMI-1640 culture media. The leukocytes' ability to migrate towards chemoattractants in TL and TNL human fetal membrane (FM) extracts were determined in an LMA.

number of migrated leukocytes in response to the various chemoattractants and 0% migration in response to DMEM, our negative control.

Moreover, the 96-well chemotaxis chamber was much easier to wash than the 48-well chemotaxis chamber. As opposed to the 48-well chemotaxis chamber measurements at times, there were continued measurements of 0% migration of leukocytes in response to the negative control at the 30-minute incubation time point. Furthermore, from a technical standpoint the 96-well chemotaxis chamber was easier to handle because the filters were manufactured with a frame, reducing the chance for human error when placing the filter on top of the lower chamber; with the 48-well chemotaxis chamber, two forceps had to be used to gently place the filter on top of the chamber, and if it was misplaced initially and then moved slightly, there would be a chance of cross-contamination between the wells containing different chemoattractants.

Chemotaxis Assay – Under-Agarose Assay. Migration was measured every 15 minutes until evidence of significant migration as measured by the % of leukocytes that were chemoattracted out of the original well. However, leukocytes tended to get stuck on the edge of the well and be unable to transition from a liquid to solid medium. As well, the agar tended to lift from the bottom of the culture plates, leading to spillage of the chemoattractants or leukocytes after loading.

Leukocyte Quantification – Fluorescence Spectroscopy. A representative standard curve, plotting the relative fluorescence units corresponding to the number of leukocytes, was generated using known concentrations of human and mouse leukocytes incubated with Hoechst 33342 (Fig. 8).

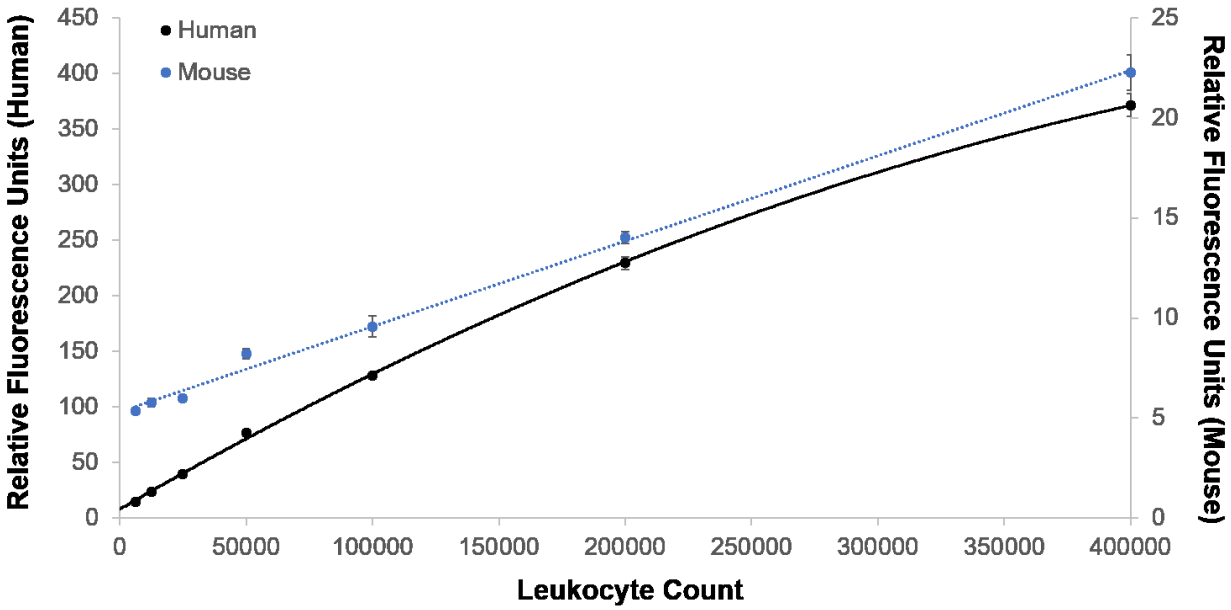

Figure 8. 4 parameter logistic (4PL) regression of standardized human leukocyte (hL) counts to relative fluorescence units. Total leukocytes were isolated from human (n=1) and mouse whole blood (n=1) samples. A serial dilution (2:1) of human leukocytes (hL) ranging from 400,000 to 3250 cells were prepared, incubated with Hoechst 33342 dye (100 µM, 350 nm/461 nm) for 20 min., and measured using an Ascent Fluoroskan in triplicate determination. Elisaanalysis.com was used to perform 4PL regression analysis. R^2<0.990 for all curves.

The standard curves had a coefficient of determination >0.990, and they were linear for readings below 200,000 leukocytes.

Furthermore, a comparison of three counting methods was used to compare the accuracy and precision of fluorescence spectroscopy as a way of quantifying leukocytes compared to flow cytometry (the method used in the original LMA) and hemocytometry (Fig. 9). There was no statistical difference between measured values as evaluated by two-way ANOVA. The precision was better for fluorescence spectroscopy than the other two methods as evaluated by lower standard mean error.

2.4. Discussion. To streamline the LMA for future clinical implementation and efficient use of laboratory resources, I identified 3 stages in the LMA for improvement: 1) leukocyte isolation, 2) chemotaxis, and 3) quantification. Overall, we identified two ways for improving the LMA's performance characteristics: using a 96-well chemotaxis chamber to resolve the chemotaxis step and using fluorescence spectroscopy to rapidly quantify Hoechst 33342-labelled leukocytes for the final quantification step, and incorporated these methods into the LMA to generate the new LMA protocol in Fig. 6. These modifications reduced the cost per assay (full 96-well plate vs full 48-well plate) by over 80% and reduced the length of the assay by over 60%.

The leukocyte isolation step could be further partitioned into three 20-minute segments: separating the leukocytes from the erythrocytes, washing and centrifugation, and counting the resuspended leukocytes. It was difficult to improve these steps without sacrificing the integrity of the isolation process. An option that we explored for this step was the MACSxpress neutrophil isolation kit under the initial assumption that at the end of the 15 minute isolation procedure, we would be left with a solution of purified neutrophils that could be used right away in a

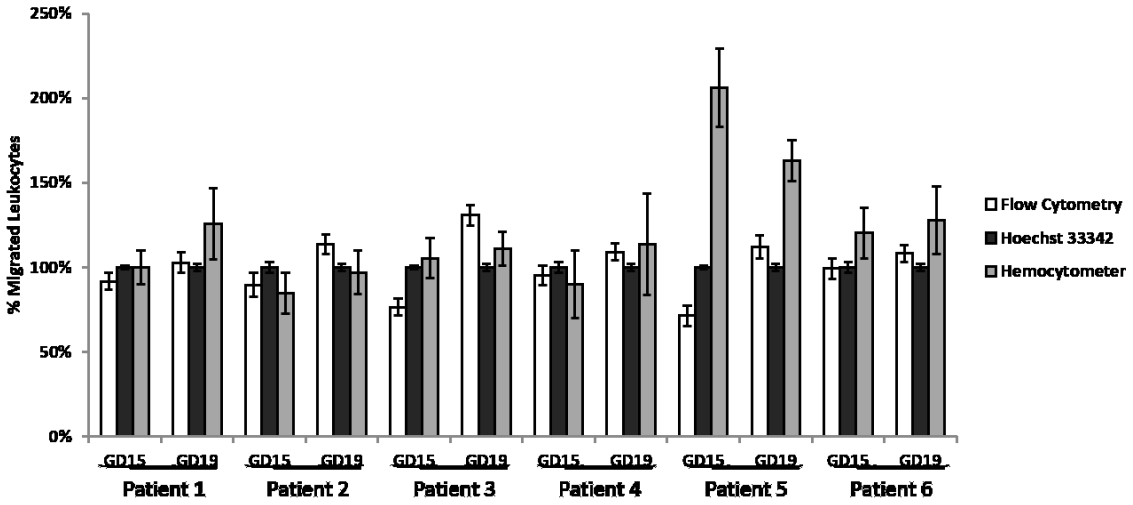

Figure 9. Comparison of three counting methods for quantifying chemoattracted leukocytes. TL hL was isolated from whole blood (n=6). GCF was extracted from mouse lower uterus from GD15 and GD19 and pooled (n=6 per group). A chemotaxis assay was used to compare the quantifications of chemoattracted TL hL in response to either GD15 or GD19 pooled GCF using flow cytometry with counting beads, fluorescence spectrophotometry with Hoechst 33342, and light microscopy with a hemocytometer in triplicate determination. Error bars indicate the standard mean error of the triplicate determinations. There was no significant difference between the measured values across the counting methods. Statistical significance was performed using two-way ANOVA.

chemotaxis assay; however, we discovered that these neutrophils had to be washed before they demonstrated activity, meaning that this step would not improve the speed of the LMA overall while costing 60x more per sample. It is unknown why these neutrophils need to be washed before they demonstrate chemotactic activity. One possibility is that the presence of antibodies to negatively select for non-neutrophils during the isolation procedure somehow inactivated the neutrophils or made their chemotactic receptors inaccessible, and the washing procedure restored them. Alternatively, the buffers used during the isolation procedure may be inhibitory to chemotaxis. There are several chemical compounds that are inhibitors of neutrophil chemotaxis, but this theory cannot be confirmed without inside knowledge of each buffer composition.

Another option for leukocyte isolation is density separation using a ficoll gradient, but it requires a similar cost and length of time per assay and will likely not be any better of a method than HetaSep. As for potential improvements to the washing step, one suggestion could be to increase the speed of centrifugation, but it risks activating the leukocytes artificially. In addition, a potentially faster, cheaper, and more accurate quantification method to count the isolated leukocytes is to use a DNA-binding cell-permeable fluorescence dye (e.g. Acridine Orange or Hoechst 33342) and automated fluorescence cell counter (e.g. Countess II FL Automated Counter). Chemoattracted leukocytes can be incubated with the dye as per the manufacturer's protocol, loaded onto a disposable slide, and inserted into the automated cell counter for rapid quantification.

To improve the chemotaxis step of the LMA, we upgraded to a larger 96-well system with wells roughly twice the diameter of the original: the 96-well chemotaxis chamber used larger wells of 18 mm^2 surface area compared to 8 mm^2 in the 48-well chemotaxis chamber. The larger surface area may have contributed to our ability to resolve the chemotaxis step faster by

over 45 minutes, and it made cleaning much more reliable. One of the biggest problems with the original version was difficulty cleaning the lower chamber wells due to their small size. Despite cleaning the chamber as directed by the manufacturer, we found positive signals for our negative control with the 48-well chemotaxis chamber. The larger chamber system therefore improved the accuracy of the assay, reduced the inter-assay and intra-assay variability, and sped up the cleaning procedure. Finally, the larger system was easier to assemble and disassemble, which also helped to reduce the time it took to perform this step.

Our last challenge was to simplify the quantification step. This was the longest and most expensive step of the assay, requiring several hours to prepare the flow cytometry tubes and many more to run them through the flow cytometer. The main advantage of using flow cytometry was being able to analyze the subpopulations of leukocytes in each sample, but this is unnecessary for diagnosing a patient for PTB risk using the LMA. A rapid method of quantifying total leukocytes would greatly improve the performance characteristics of the LMA. An alternative option that we briefly explored for improving the quantification step included size based automated cell counting, but unfortunately results for the size-based automated cell counting were unreliable due to red blood cell contamination. Fluorescence spectrometry, however, proved a cheap, efficient solution for quantifying total leukocytes. We tested and validated Hoecsht 33342, a DNA-binding dye that is excited at 350 nm and emits at 461 nm.

More work is needed to improve the assay further, and one direction that development can take is to design a simpler chemotaxis apparatus. With the help of University of Alberta Libraries, I used a Machina mk2 3d-printer (Machina Corp, Edmonton, AB, Canada) to print a single point chamber with the diameter and depth of the original chemotaxis chamber so that it could be easily assembled and disposed of afterwards (Appendix 1). Preliminary testing failed

due to a lack of integrity of the filter staying on the upper chamber during disassembly of the apparatus, but a disposable, LMA-specific chemotaxis system may be worth looking at. Particularly if the LMA is to be used in clinic, using a disposable system would allow for a higher level of consistency between tests, and costs may even be saved from the time and labour it would take to clean the system. Moreover, having single-point or triple-point systems would allow for individual patient samples to be run simultaneously without danger of cross-contamination between wells, and clinicians would not have to wait for multiple patients before running a plate.

Another possible direction for LMA development is improving the quantification method. Using a fluorescence plate reader and 4PL regression analysis may be efficient in the research setting, but it may not be ideal for clinical implementation. The reason for this is that an internal standard curve is advisable for every patient given that hemocytometry, the technique used to count and seed the initial concentration of leukocytes in the top well of the chemotaxis chamber, is unreliable. Generating an internal standard curve for every patient is inefficient for clinical practice. An improved quantification method could use be to use a DNA-binding cell-permeable fluorescence dye (e.g. Acridine Orange or Hoechst 33342) and automated fluorescence cell counter (e.g. Countess II FL Automated Counter) as described earlier for the quantification of isolated leukocytes step of the assay.

Chapter 3. Leukocyte Invasion in Mouse Parturition.

3.1. Introduction. Leukocyte invasion is a parturition phenomenon that is conserved among humans, mice, rats, and guinea pigs [3-5]. However, the mechanisms that regulate the targeting and timing of leukocyte invasion are not completely understood. These two outcomes are highly important: if leukocyte invasion is undirected, these highly active leukocytes could cause rampant invasion in vulnerable tissues and initiate systemic inflammation, as it could happen during infection-associated preterm birth (PTB); and if the timing of leukocyte invasion is not regulated, the subsequent pro-labor events that these invading leukocytes facilitate may not occur soon or late enough to ensure that parturition occurs at an appropriate time.

3.1.1. Regulation of Leukocyte Invasion by Gestational Chemotactic Factors. The targeting and timing of leukocyte invasion may be regulated by an increase in gestational chemotactic factors (GCF) at the target site leading up to labour. This has been suggested by studies that demonstrate in humans that leukocyte invasion is associated with an increased myometrial expression of the major neutrophil chemoattractant interleukin (IL)-8, and that total GCF extracted from TL human fetal membranes (hFM) can stimulate *in vitro* leukocyte chemotaxis more than TNL hFM [56, 238]. Moreover, in guinea pigs and rats, the secretion of GCFs in amniochorion and uterus increase throughout late gestation respectively [3, 5]. Mouse GCF has not yet been characterized. Interestingly, the specific tissues that demonstrate increased GCF differ from one species to another; this suggests that the target of leukocyte invasion may also vary between species.

3.1.2. Regulation of Leukocyte Invasion by Enhanced Leukocyte Chemotaxis. Another mechanism that regulates the timing of leukocyte invasion is the enhanced ability of leukocytes to migrate towards GCFs that occurs near labor onset. Peripheral human leukocytes during term or preterm labour exhibit enhanced chemotaxis towards a standard chemoattractant compared to TNL or PTNL leukocytes [61]. Rats also exhibit parturition-associated enhanced leukocyte chemotaxis, evidenced by >10x activity at GD22 than GD17 [3]. Whether mice exhibit this phenotype is unknown. It is thought that enhanced leukocyte chemotaxis and increased GCF work in conjunction to achieve leukocyte invasion during parturition.

3.1.3. Leukocyte Invasion and PTB. In the context of PTB, leukocyte invasion is a controversial phenomenon. Although a large influx of neutrophils into the decidua and myometrium has been detected in a LPS-induced mouse model of PTB [86, 87], the Norman group demonstrated that antibody-based neutrophil depletion does not alter the timing of birth nor prevent LPS-induced PTB [239]. In this latter study, it was concluded that neutrophils were unnecessary for PTB. Instead, neutrophils were suggested to play a role in postpartum repair and remodeling of the uterus and cervix [86, 87]. Alternatively, the findings from the Norman study could suggest that the LPS used to induce PTB serves a similar role as the neutrophils that are recruited to the gestational tissues; in other words, the neutrophils that were depleted may have been redundant following LPS administration.

There are limitations to the Norman study, including the likelihood that neutrophil depletion results in abnormal physiology. Other cells may be able to compensate for the loss of neutrophil activity. For example, neutrophils produce MMP9 which is involved in fetal membrane breakdown, but it can also be produced by local macrophages [71]. Furthermore,

neutrophil depletion could provoke the onset of PTB itself: the depletion of neutrophils in LPS-treated mice significantly decreases the time to delivery [239]. Further study is needed to understand the relationship between PTB and leukocyte invasion.

Now is the time to study this relationship, and particularly in the mouse. Recently, intrauterine IL-1β administration in mice has been reported to induce PTB and an increase in pro-inflammatory cytokine expression in uterine tissues, suggesting that these tissues are inflamed [240]. This mouse model offers investigators the opportunity to study the relationship between PTB and leukocyte invasion. Moreover, a novel allosteric modulator of the IL1R, 101.10, was recently introduced as a potential PTB therapeutic. 101.10 is also called RYTVELA, the 7 amino-acid sequence that comprises the drug (Fig. 10). The half-life of 101.10 is 12 hours, and 101.10 is a non-competitive inhibitor of the IL1R1 [241]. 101.10 is also a preventative agent for both infection- and inflammation-associated PTB in the mouse model, and can differentially modulate signaling pathways induced by IL-1β by inhibiting IL1-activated SAPK/c-Jun and Rho-ROCK pathways while leaving NF-κB activity intact [240]. This gives 101.10 an advantage over orthostatic antagonists because it preserves the protective effects of the NF-κB pathway, including 1) host defense during and following parturition; 2) cytoprotection and the preservation of epithelial barrier integrity; and 3) apoptotic termination of an inflammatory response [176]. 101.10 has also been demonstrated to reverse the increased mRNA abundance for proinflammatory cytokines in the fetal brain and lung of PTB-induced mice, suggesting that it has feto-protective effects [212]. The development of this drug is timely, because 101.10 enables us to confirm whether the relationship between PTB and leukocyte invasion is dependent on IL-1β.

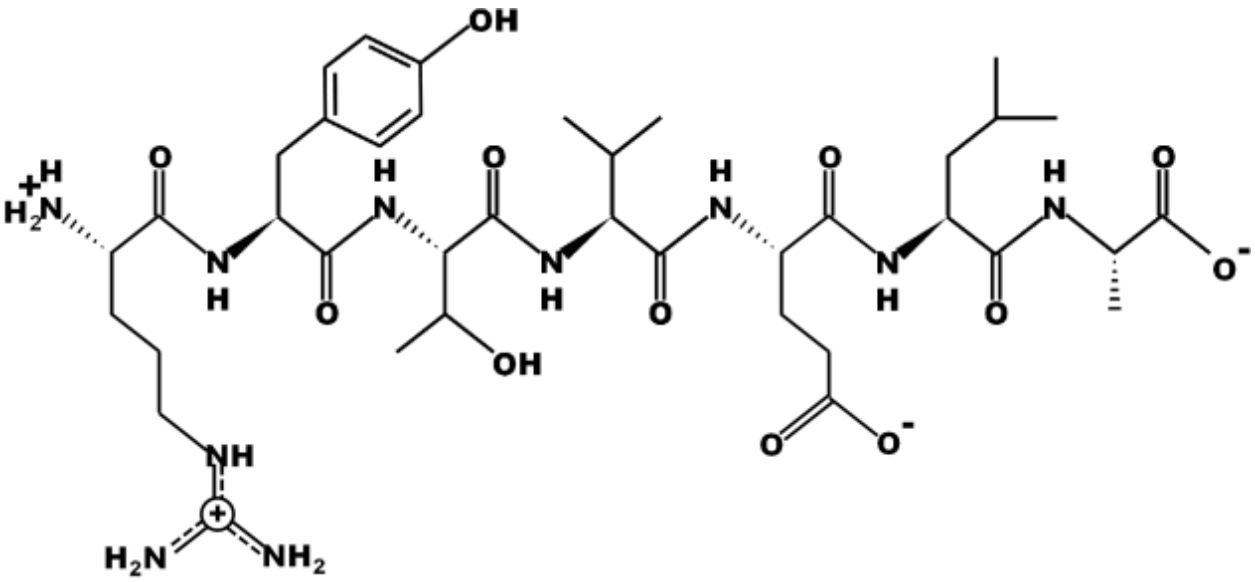

Fig. 10. Structure of 101.10 (aka. RYTVELA). Drawn using PepDraw by Thomas C. Freeman.

http://pepdraw.com/

3.1.4. Rationale, Hypotheses, Experimental Approach. The intent of the present study was to achieve a better understanding of parturition- and PTB-associated leukocyte invasion and the mechanisms underlying its regulation. This information is important for several reasons: it could 1) provide direction for future PTB diagnostics and therapeutics that may seek to target upstream pathways of leukocyte invasion; 2) help identify a mechanism by which IL-1β can induce PTB; 3) help identify a mechanism by which 101.10 can protect a pregnancy from PTB stimuli; and 4) offer insight into whether maternal IL-1β can lead to fetal inflammation and whether 101.10 can prevent this effect. This last determination could be significant for the development of 101.10 as a PTB therapeutic, since preventing spontaneous fetal expulsion by itself may not be enough to demonstrate an improvement to perinatal outcomes; if inflammation of fetal tissues can also be prevented by 101.10, it could additionally serve as a feto-protective agent from fetal inflammatory disease and be a better candidate as a PTB therapeutic.

The two mechanisms that we focused our attention on was the regulation of leukocyte invasion by an increase in GCF at the target tissues in mice and by an enhanced chemotaxis phenotype in mouse peripheral leukocytes. To the study the GCF, we first sought to compare the GCF in various mouse tissues at multiple points throughout late gestation to determine if leukocytes might be being targeted to specific tissues as the dam approached labor and to determine when the secretion of GCF occurs. We hypothesized that GCF would be highest in the gestational tissues that were being targeted and that the timing of GCF secretion would begin just before labor and continue to rise until labor. Second, we sought to determine whether this regulation of leukocyte invasion occurs in a mouse model of IL-1β-induced PTB; to do this we looked at whether leukocyte invasion occurs at all in our model and then compared the GCF in the target gestation tissues before and after preterm labor induction. We hypothesized that

neutrophil density would increase in mouse gestational tissues, and that this would be associated with an increase in GCF at the target tissues.

We then studied whether peripheral leukocytes were enhanced for chemotaxis in this mouse model by comparing leukocyte activity before and after preterm labor induction, and followed through with an experiment to study whether pre-treatment of TNL leukocytes (which are not as active as TL leukocytes [4]) with maternal serum factors at TL could artificially induce the enhanced chemotaxis phenotype, to begin the discussions regarding where the enhancement of leukocytes takes place physiologically. We hypothesized that peripheral mouse leukocytes would be more responsive to a standard chemotactic factor after labor induction with IL-1β, and that we could replicate the enhanced leukocyte chemotaxis phenotype with TL maternal serum factors but not TNL maternal serum factors.

In addition, we determined whether leukocyte invasion also occurs in the fetuses of this model by comparing the number of neutrophils in the fetal brain and lung before and after preterm labor induction, two tissues that have previously been characterized as being abundant for pro-inflammatory cytokine mRNA [240]. We also studied whether this effect occurs during lipopolysaccharide (LPS)-induced PTB in mice, a well-established infectious model of PTB. We hypothesized that neutrophil invasion occurs in these tissues.

Finally, we studied the effects of 101.10 on this multitude of potential IL-1β-mediated effects in this mouse model. We hypothesized that 101.10 co-treatment would be able to reverse these effects.

3.2. Methods. *Experimental Groups*. Timed-pregnant mice were euthanized at GD15, 17, 18, 18.5, 19, and post-partum (PP)-1. Other timed-pregnant mice at GD16 were anesthetized under

isoflurane and injected with either IL-1β (3 μg) or vehicle (0.9% saline) in the right horn of the mouse lower uterus between two fetal membranes. These mice were injected with 101.10 (1 mg/Kg/12h) or vehicle (0.9% saline) subcutaneously in the neck 30 minutes before IL-1β administration. Mice were expected to deliver on GD19. The treatment groups are described in Table 4.

Lower uteri (LU), upper uteri (UU), placentas (PL), fetal membranes (FM), cervixes (CX), and fetuses were collected as necessary and normalized to wet tissue weight in Dulbecco's Modified Eagle's Medium (DMEM). GCF were extracted via homogenization and centrifugation to get rid of the cellular debris. Mouse whole blood was collected via cardiac puncture, and total leukocytes were isolated using HetaSep according to the manufacturer's protocol.

TL human leukocytes (hL) were isolated from whole blood as described in the methods section of Chapter 2.

Leukocyte Migration Assay. The LMA was performed as described in the methods section of Chapter 2. In brief, TL hL were loaded into the top wells of a 96-well chemotaxis chamber system and chemoattractants were loaded into the bottom wells. TL hL were incubated at 37ºC in 5% CO_2 for 30 minutes to undergo chemotaxis through a porous polycarbonate filter (3 μm diameter pores). Chemoattracted leukocytes were labelled with Hoechst 33342, a fluorescent DNA-binding dye, and quantified using a fluorescence spectrophotometer.

Immunohistochemistry. Six cryosections (7 μm) per treatment group were prepared from snap-frozen mLU and stained for neutrophils using a goat anti-mouse Ly6G antibody and a rat anti-goat secondary antibody tagged with an Alexa Fluor 488 fluorescence marker. Ly6G is a 21-25

Table 4. Experimental Treatment Groups.

	GD17 Control	GD18.5 Control	GD17 Sham	GD17 IL-1β + veh	GD17 IL-1β + 101.10	GD17 101.10 alone
ED[1]	17	18.5	17	17	17	17
IL-1β	-	-	veh[2]	+	+	veh
101.10	-	-	veh	veh	+	+

[1]ED: euthanization day
[2]veh: vehicle (saline)

kDa glycosylphosphatidylinositol (GPI)-linked differentiation antigen that is expressed on the cell surface of neutrophils. Cells were counterstained using Hoechst 33342 (Thermo Fisher Scientific, MA, USA) and tissue homology was confirmed with an H&E stain. Four different fields (20x optical zoom) were counted per section by two observers blinded to the specimen details. Areas containing blood vessels and leukocytes within blood vessels were excluded. The arithmetic mean was calculated for each sample between the two observers.

Isolation of RNA. RNA was extracted from mouse whole blood using the RNeasy protect animal blood system (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In brief, blood samples were mixed with a reagent that lyses erythrocytes and stabilizes intracellular RNA. The reaction is stopped and proteins are digested with a proteinase under homogenization by centrifugation in spin columns to eliminate cellular debris. Ethanol is added to precipitate RNA and DNA is degraded using DNase. After several washing steps, pure RNA is then eluted using the manufacturer's elution buffer. RNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer to measure the optical density (OD) photometrically at 280 nm and 260 nm (an $OD_{260nm/280nm}$ ratio of >1.8 was considered protein-free RNA [273]).

RT-qPCR. cDNA was synthesized from 500 ng RNA using qScript^{TM} cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA) according to the manufacturer's protocol resulting in a total reaction volume of 20 µL, which was used in quantitative polymerase chain reactions (qPCR). Primers for mouse *il-1β*, *il-6*, *tnf-α*, and *ccl2* were designed using the National Center for Biotechnology Information's Primer Blast. Primer sequences are described in Table 5. The annealing temperature of all primers was 60ºC.

Table 5. RT-qPCR Primers for Mouse Cytokines.

	Forward Primer	Reverse Primer
IL-1β	5′-AGATGAAGGGCTGCTTCCAAA-3′	5′-GGAAGGTCCACGGGAAAGAC-3′
IL-6	5′-CAACGATGATGCACTTGCAGA-3′	5′-TCTCTCTGAAGGACTCTGGCT-3′
TNF-α	5′-GCCTCTTCTCATTCCTGCTTG-3′	R: 5′-CTGATGAGAGGGAGGCCATT-3′
CCL2	5′-GCTCAGCCAGATGCAGTTA-3′	R: 5′-TGTCTGGACCCATTCCTTCT-3′

Quantitative gene analysis was performed using SYBR Green Master Mix (Bio-Rad) according to the manufacturer's protocol. Each 20 µL reaction was run with duplicate determination and included 1 µL cDNA (25 ng), 10 µL of 2x PerfeCTa SYBR Green FastMix for iQ (Quanta Biosciences), 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, and 8 µL H_2O. Using the *i*Cycler IQ equipment and software (Bio-Rad Laboratories, Hercules, CA, USA), 2-step qPCR was performed using the following parameters: 10 min at 95ºC, followed by 45 cycles of 15 seconds at 95ºC and 1 minute at 60ºC (annealing temperature). Following amplification, melt curve analysis was performed to ensure that the amplification of non-specific products did not occur. PCR products from the primers used in this study have been previously confirmed by gel electrophoresis on a 2% agarose gel, and dissociation curves were also acquired to confirm primer specificity by Nadeau-Vallee *et al* [97]. Standard curves for both the target genes and mouse β-actin (Actb) were generated by serial dilutions of the cDNA samples, and analyzed using the *i*Cycler. Amplification efficiencies for each forward and reverse primer set were determined manually by converting the slope of the standard curve using the equation $E = 10^{-1/slope}$. The mean threshold cycle value was calculated for each reaction, corrected for the amplification efficiency, and then expressed relative to the negative control group (GD17). Target gene levels were expressed relative to *actb*.

Leukocyte Incubation with Maternal Serum. TL or TNL whole blood was left to clot at RT for 30 minutes, after which serum was isolated by centrifugation at 1500 x *g* for 10 minutes at 4ºC. The serum was stored at -80ºC immediately upon collection. $1x10^7$ TNL hL were incubated with the serum at a 1:1 ratio for 1 hour at RT under gentle agitation using a stir bar. The TL hLs were precipitated via density centrifugation and resuspended in RPMI. An LMA was used to assess

the responsiveness of hL upon stimulation with TL or TNL maternal serum or IL-8 (1 μg/mL) compared to control.

3.3. Results. *GCF increase in the Mouse Lower Uterus and is Highest at GD18.5.* Next, a longitudinal study was conducted to characterize the pattern of GCF in mouse gestational tissues throughout late gestation. Briefly, GCF was extracted from GD15, GD18.5, and GD19 mouse LU, UU, PL, CX, and FM, and compared using TL hL in an LMA. The response of TL hL was significantly higher for LU GCF at GD18.5 than GD15, whereas it was significantly lower for FM and CX GCF in mice that were closer to spontaneous labour (Fig. 11). A closer look at the pattern of GCF in the mLU demonstrated that the chemotactic response of TL hL to GCF peaked at GD18.5 and fell post-partum (Fig. 12).

Ly6G+ Neutrophils Invade the Mouse Lower Uterus after PTB Induction. The schematic we used to study the regulation of leukocyte invasion in the context of PTB is detailed in Fig. 13. First, we confirmed that intrauterine administration of IL-1β on GD16 induces PTB in the CD1 mouse. Mice were monitored at 30-minute intervals until the PTB, which occurred within 24 hours of administration of the labour-inducing agent in 85% of mice (Fig. 14A). A comparison of the fetuses removed post-euthanization on GD17 demonstrated that after IL-1β-induced PTB the fetuses look much smaller and less developed than the control (Fig. 14B). These effects were mitigated with 101.10 co-treatment. Then, IHC was used to quantify Ly6G+ neutrophil density in the mLU. IL-1β-induced PTB was associated with an increased detection of Ly6G+ neutrophils in the mLU, and a similar increase was also detected at GD18.5 in untreated mice (Fig. 14C, D). Co-treatment with 101.10 reversed the effect of IL-1β.

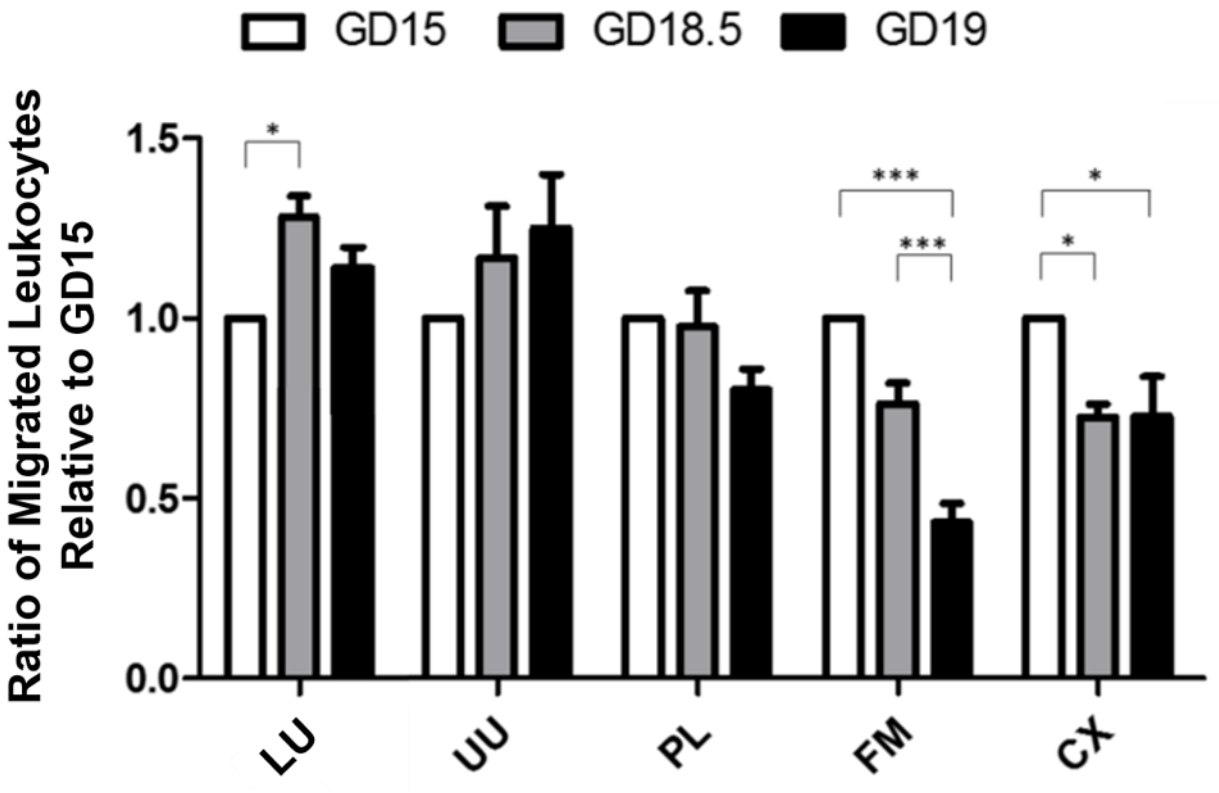

Figure 11. Comparison of gestational chemotactic factor from mouse tissues: lower uterus (LU), upper uterus (UU), placenta (PL), fetal membrane (FM), and cervix (CX). GCF was pooled from GD15, GD18.5, and GD19 mice (n=6). An LMA was used to measure TL hL response to these GCF. Statistical significance was performed using two-way ANOVA, and Tukey's HSD test as a *post hoc* test. *$p<0.05$, ***$p<0.001$.

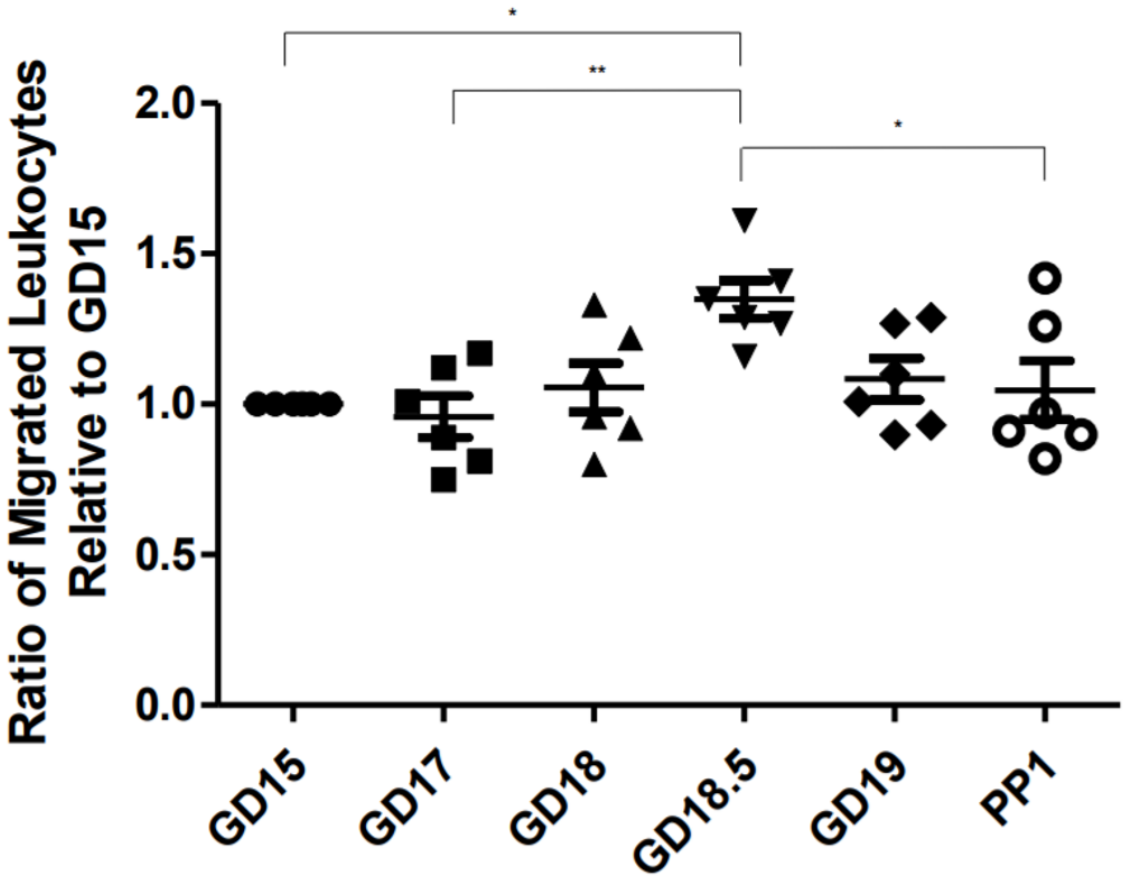

Figure 12. Changes to gestational chemotactic factor in the mLU with increasing gestation. GCF was pooled from GD15, GD17, GD18, GD18.5, labor at GD19, and one day post-partum (PP1) mice (n=6). An LMA was used to measure TL hL response to these GCF. Statistical significance was performed using one-way ANOVA, and Tukey's HSD test as a *post hoc* test. *$p<0.05$, **$p<0.01$.

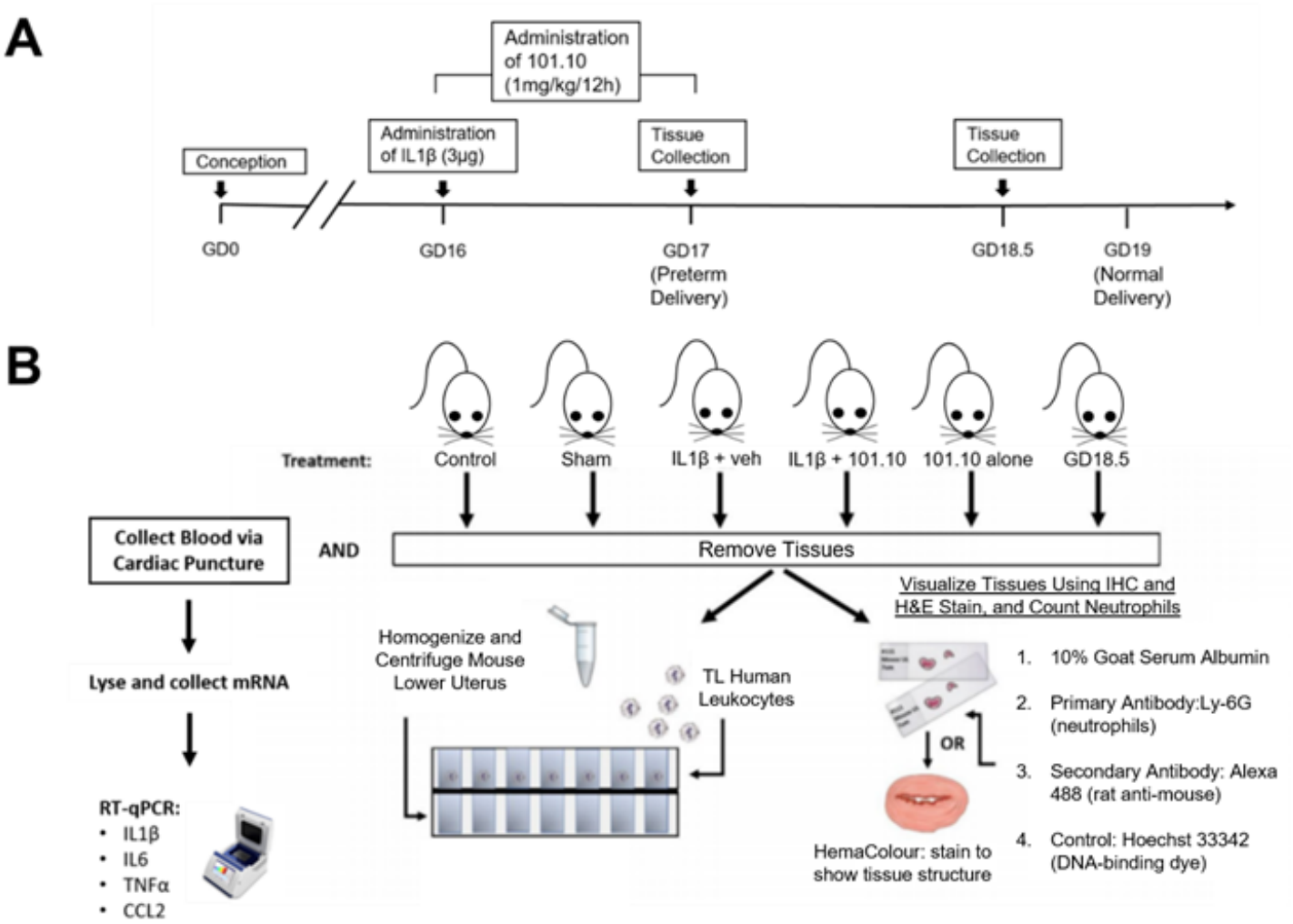

Figure 13. Schematic for the collection and processing of mouse tissues for extracting gestational CF, visualizing neutrophils, and isolating leukocyte mRNA. (**A**) Experimental timeline. (**B**) Experimental schematic. Credit for diagram: Meghan Onushko.

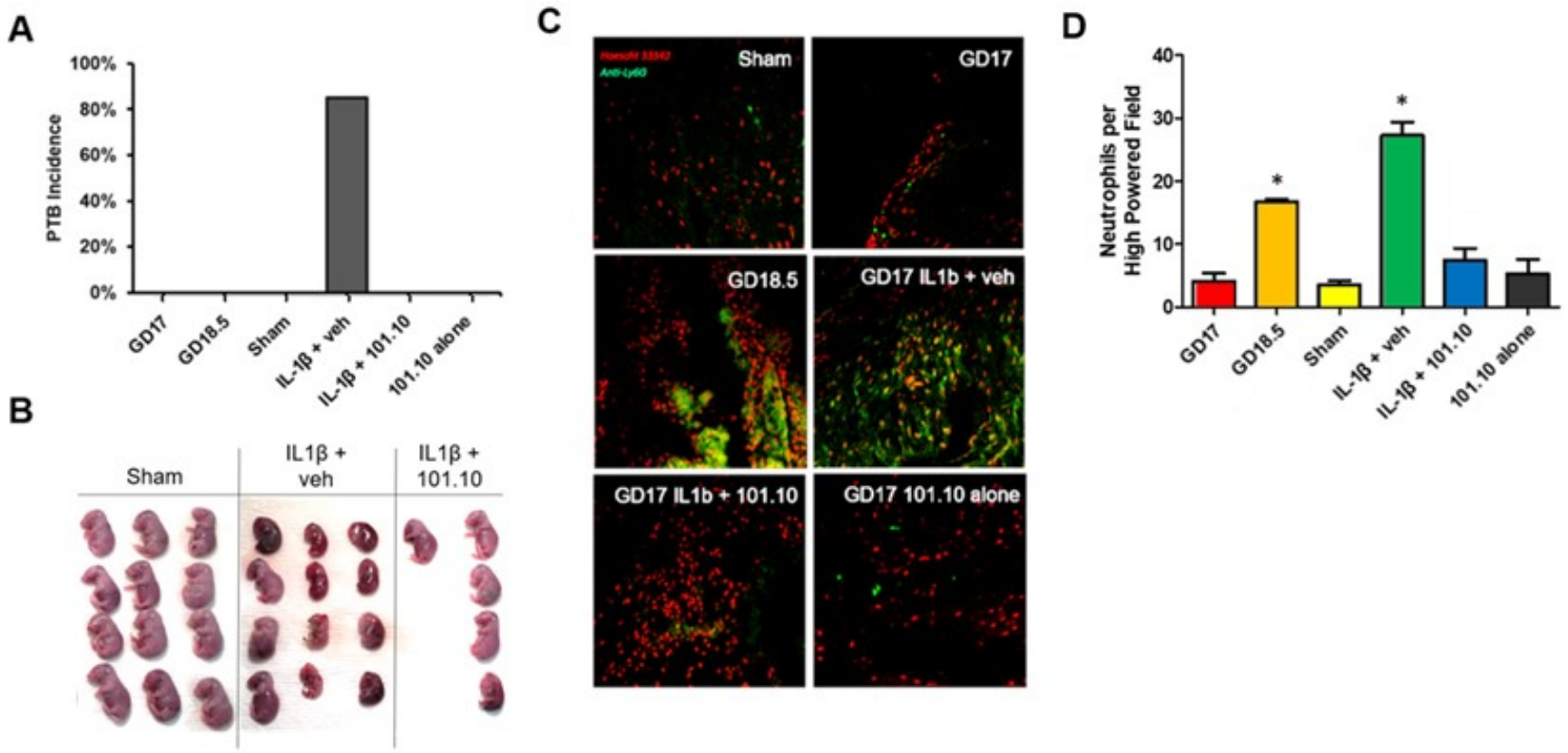

Figure 14. Neutrophil invasion of the mLU during late gestation and IL-1β-induced PTB. (**A**) PTB incidence in mouse treatment groups (n=5). (**B**) Images of GD17 fetuses, 24 hours after injection of the labor-inducing agent, with or without 101.10, or vehicle. (**C**) Representative histological images of the mouse lower uterus. (**D**) Counts of neutrophils per high powered field (20x optical zoom) in mouse lower uterus stained for DNA (Hoechst 33342, red stain) and a neutrophil surface marker (anti-Ly6g, green stain). Statistical significance was performed using one-way ANOVA, and Tukey's HSD test as a *post hoc* test. *$p<0.05$ compared to GD17.

No Increase in GCF after PTB Induction. TL hL were used in an LMA to compare the mLU GCF. There was no difference in the TL hL response to GCF from the IL-1β-treated group compared to the untreated group (Fig. 15). We also confirmed that the chemotactic response of TL hL was greater to GCF from the mLU at GD18.5 than GD17. Co-treatment with 101.10 had no effect.

Enhanced Leukocyte Chemotaxis in PTB-Induced Mice. To determine whether enhanced leukocyte chemotaxis occurs in our PTB mouse model, we isolated peripheral mouse leukocytes from whole blood, measured their mRNA abundance for IL-1β, IL-6, TNF-α and CCL2, and assessed their chemotactic response to GCF. Both untreated GD18.5 and IL-1β-treated GD17 mouse leukocytes demonstrated higher mRNA abundance for IL-1β, IL-6, TNF-α and CCL2 than untreated GD17 mouse leukocytes (Fig. 16). Co-treatment with 101.10 reversed this effect for IL-1β, TNF-α and CCL2, and 101.10 alone had no effect. In addition, more of the IL-1β-treated GD17 mouse leukocytes were chemoattracted by mLU GCF than the sham-treated GD17 mouse leukocytes (Fig. 17). Co-treatment with 101.10 successfully reversed this effect.

TL Serum Primes Human TNL Leukocytes for Chemotaxis. The results of Chapter 3 suggest that IL-1β regulates leukocyte invasion by enhancing leukocyte chemotaxis, but the mechanism in which this occurs is still ambiguous. To elucidate this mechanism, TNL hL were incubated for 1 hour with serum from TL or TNL mothers and compared using an LMA (Fig. 18). TNL hL was enhanced by both TL and TNL maternal serum to migrate towards to TL hFM GCF but not towards TNL hFM GCF nor IL-8 (Fig. 19). There was near-zero migration in response to IL-8. A

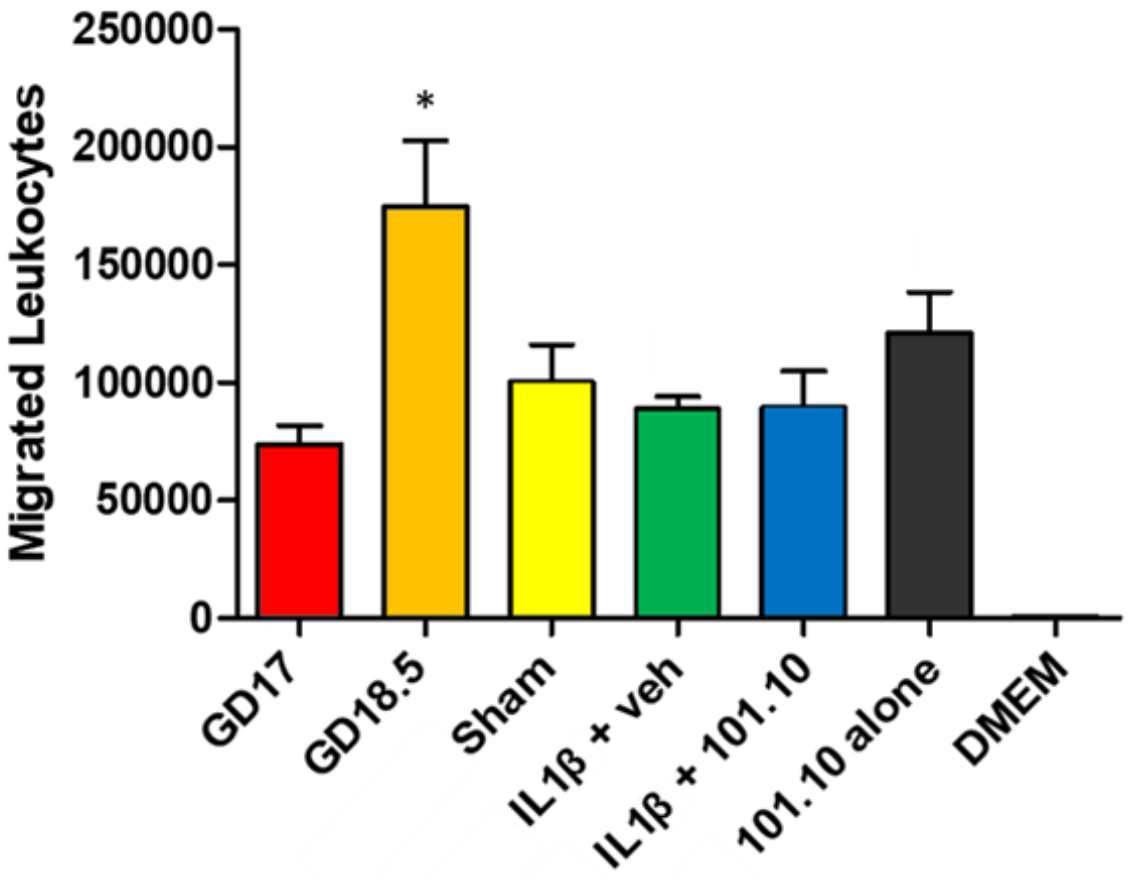

Figure 15. No effect of IL-1β treatment on gestational chemotactic factor in the mouse lower uterus. Mice were treated as described previously (n=5 to 7). GCF were extracted from the lower uteri corresponding to the indicated mouse treatment groups. An LMA was used to measure TL hL response to these GCF. Statistical significance was performed using one-way ANOVA, and Tukey's HSD test as a *post hoc* test. *$p<0.05$ compared to GD17.

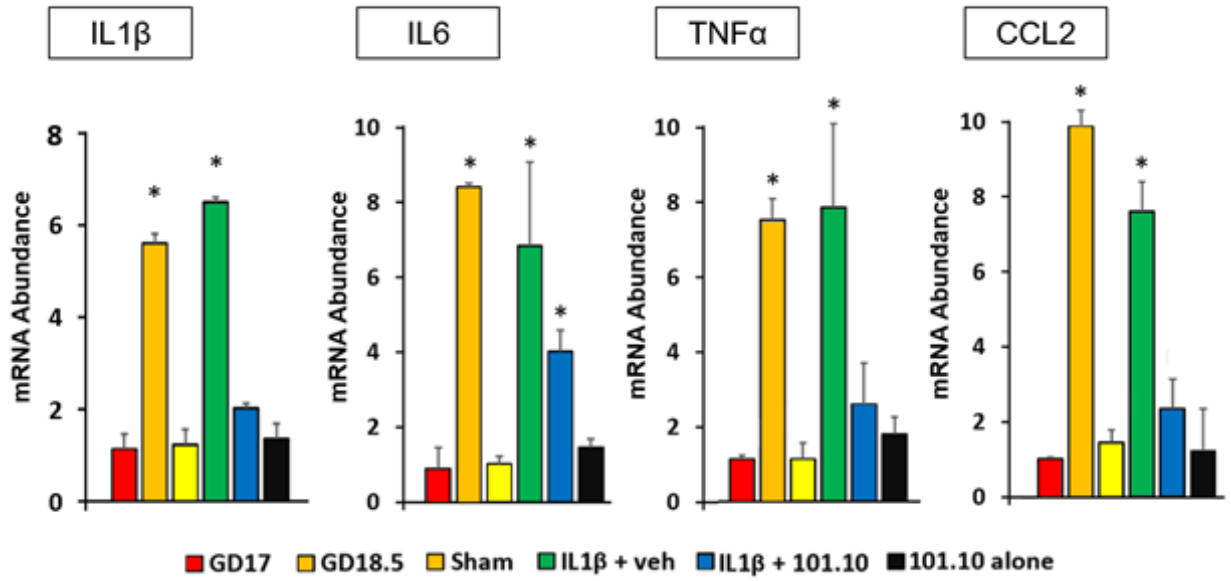

Figure 16. Cytokine expression in peripheral mouse leukocytes increases in an IL-1β-induced model of PTB. Systemic blood was collected via cardiac puncture post-partum from treated and untreated mice (n=3 per point). mRNA was extracted according to the manufacturer's protocol, and used to synthesize cDNA. Quantitative gene analysis was performed and gene levels were normalized to mouse β-actin. Melting curves were used to test primer specificity. Statistical significance was performed using one-way ANOVA for each cytokine, and Tukey's HSD test as a *post hoc* test. *$p<0.05$ compared to GD17.

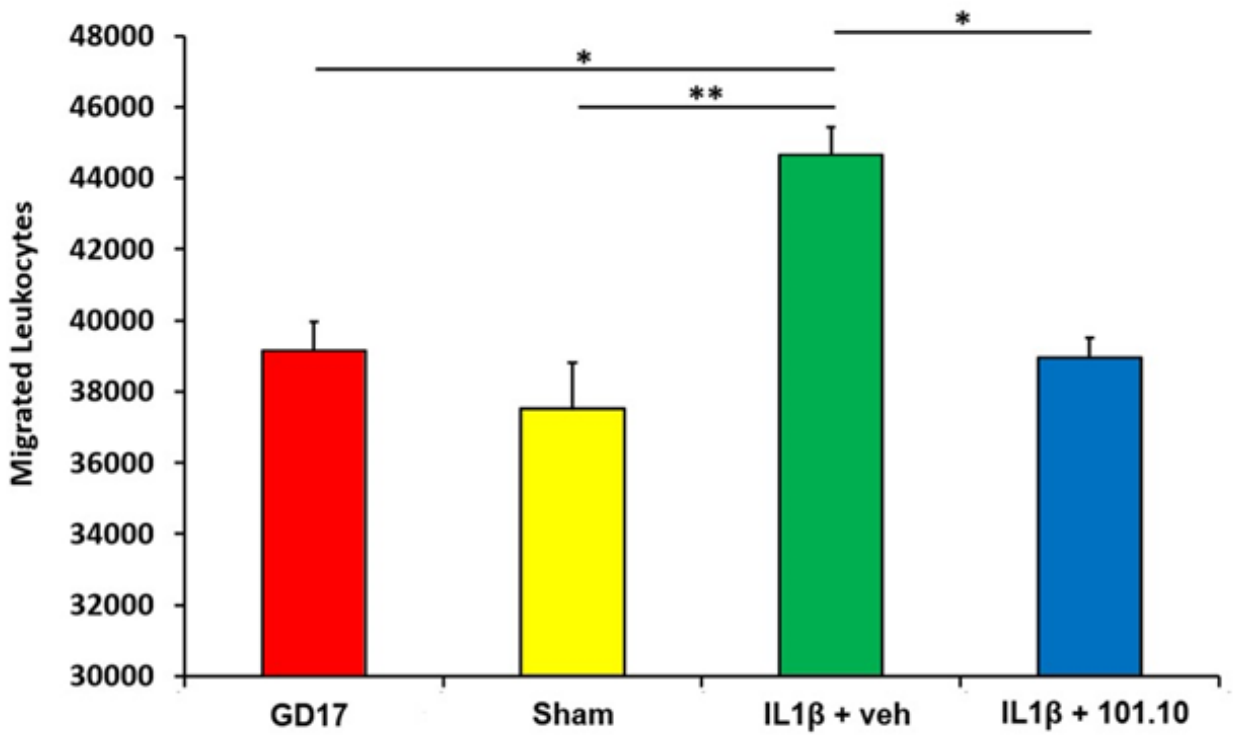

Figure 17. IL-1β treatment primes peripheral mouse leukocytes for chemotaxis to a standard chemoattractant. Peripheral leukocytes were isolated from whole blood from the indicated treatment groups (n=2 or 3). GCF was collected from mice euthanized at GD17 for the indicated treatment groups. An LMA was used to measure mouse leukocyte response to these GCF. Statistical analysis was performed with one-way ANOVA ($p<0.005$) and Tukey's HSD test as a *post hoc* test. *$p<0.05$, **$p<0.01$.

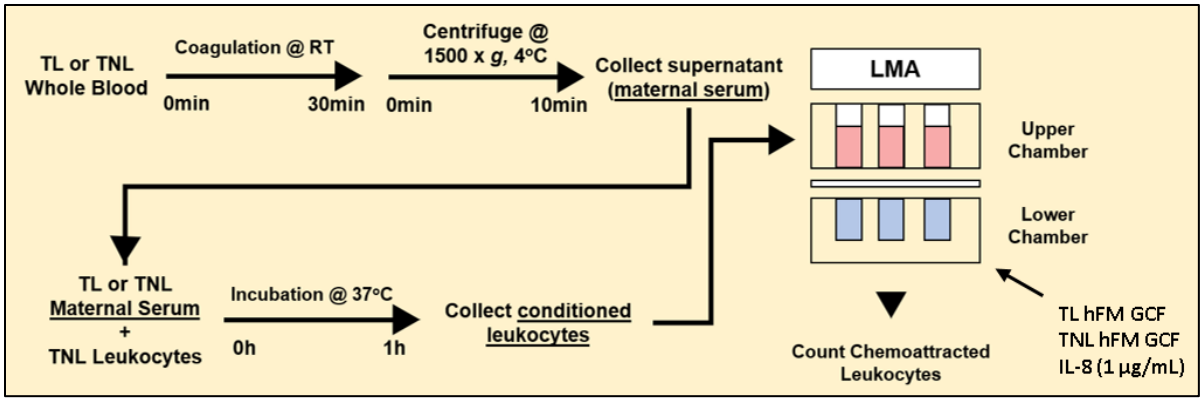

Figure 18. Schematic for assessing TNL leukocyte chemotaxis after pretreatment with maternal serum. TNL Leukocytes were isolated and incubated for 1 hour at RT under gentle agitation with TL or TNL maternal serum. Leukocytes were precipitated using density centrifugation and assessed for chemotaxis in a leukocyte migration assay in response to gestational chemotactic factors from TL or TNL human fetal membranes, or IL-8 (1 µg/mL).

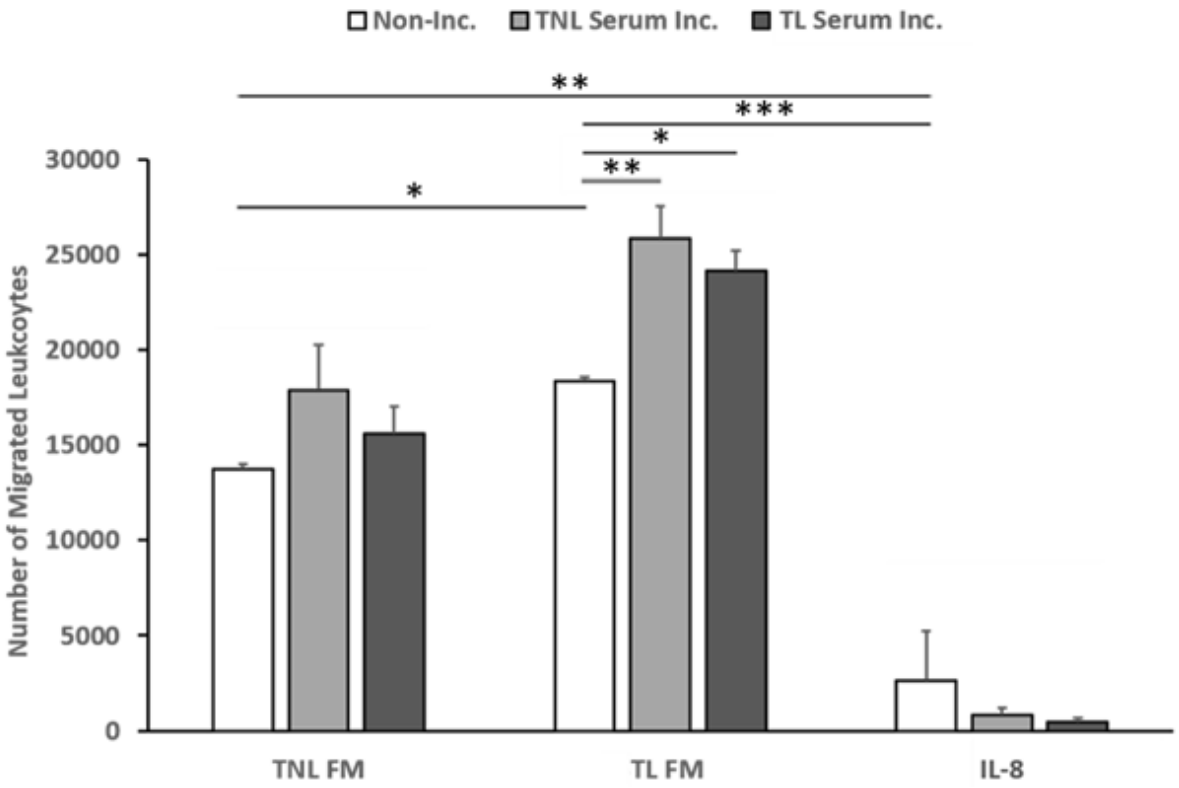

Figure 19. Maternal serum primes TNL leukocytes for chemotaxis to gestational chemotactic factor. Leukocytes were isolated from TNL whole blood and incubated (inc.) with serum (TNL: n=7, TL: n=6) or control medium for 1 hour under gentle agitation to prevent clumping. An LMA was performed using the conditioned TNL hL after washing to remove serum components. The chemoattractants used were homogenized TNL and STL hFM, and IL-8 (1 μg/mL). Statistical significance was performed using two-way ANOVA, and Tukey's HSD test as a *post hoc* test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

screen of the maternal serum for 40 major human cytokines indicated that there was an increase in CCL21, CCL11, IFN-γ, IL-4, IL-6, IL-8, CCL20, and CCL23 from TNL to TL (Fig. 20).

Ly6G+Neutrophils Invade the Fetal Brain and Lung after PTB Induction. We demonstrated that IL-1β-induced PTB in mice is associated with invasion of Ly6G+ neutrophils in the fetal brain and lung, and that this was successfully reversible with 101.10 co-treatment (Fig. 21). LPS-induced PTB in mice was also associated with Ly6G+ neutrophil infiltration of the fetal brain and lung, and 101.10 co-treatment reversed this effect.

3.4. Discussion. *GCF in Murine Spontaneous Labour.* We also report that murine GCF increases at the mLU as a dam progresses towards labor, suggesting that the mLU may be the primary target for parturition-associated leukocyte invasion in mice. This targeted infiltration could be important to labour onset in mice, because having a greater concentration of neutrophils, which are a rich source of proteins that degrade the ECM, in the mLU may ensure that the order of fetal membrane rupture occurs in fetuses closest to the cervix. Moreover, we report that the timing of this increase is specifically at GD18.5, as the response of leukocytes to mLU GCF peaks at GD18.5 and falls soon after, reaching a significantly lower level post-partum.

On the other hand, GCF in the mouse fetal membranes decreases prior to spontaneous labour. This is in stark contrast to what we find in the hFM, perhaps due to the physiological differences between the murine and primate fetal membrane structures. Rodents form an inverted visceral yolk sac that encloses the fetus and amnion and functions as an accessary to the chorioallantoic placenta through term [242]. In contrast, humans follow a second pattern in which they form a secondary yolk sac within the exocoelom [242]. The murine inverted visceral

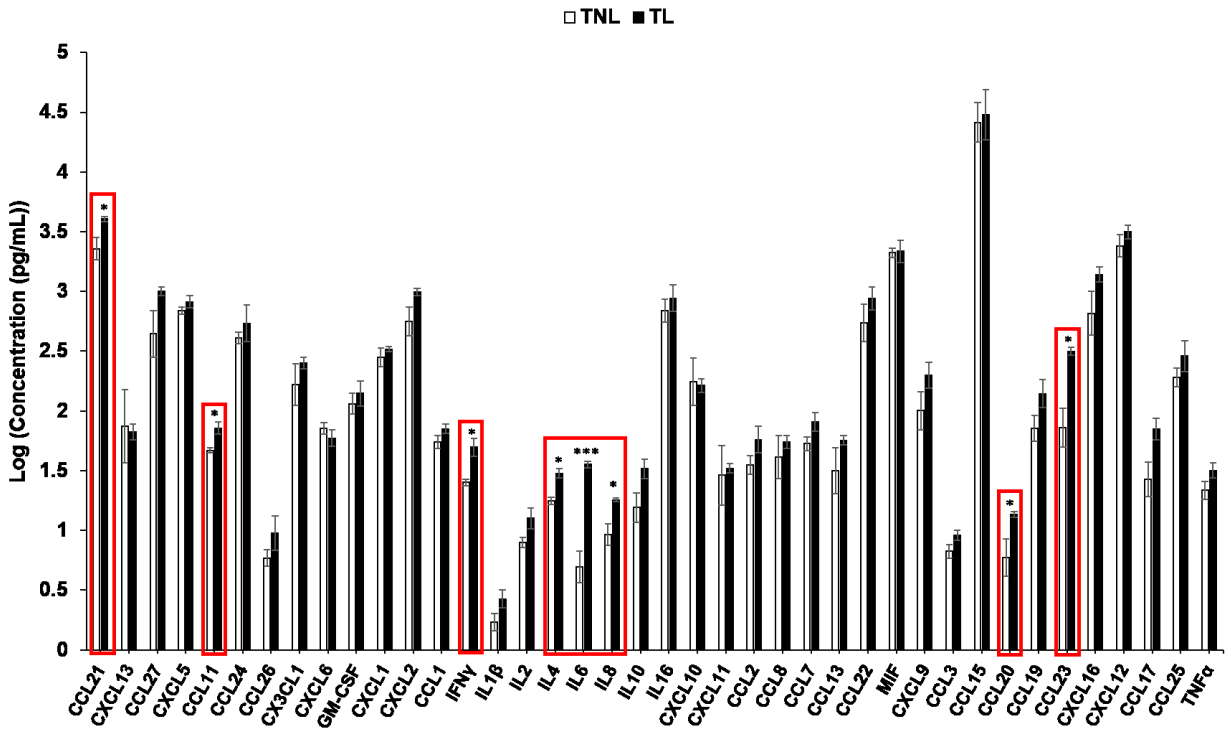

Figure 20. Comparison of 40 cytokines between TL and TNL Maternal Serum. Serum was isolated from TL and TNL whole blood (n=3) using centrifugation. Serum components were quantified using multiplex screening analysis. Statistical analysis was performed using Student's T-test. *$p<0.05$, ***$p<0.001$. Statistically significant differences are also indicated by the boxes.

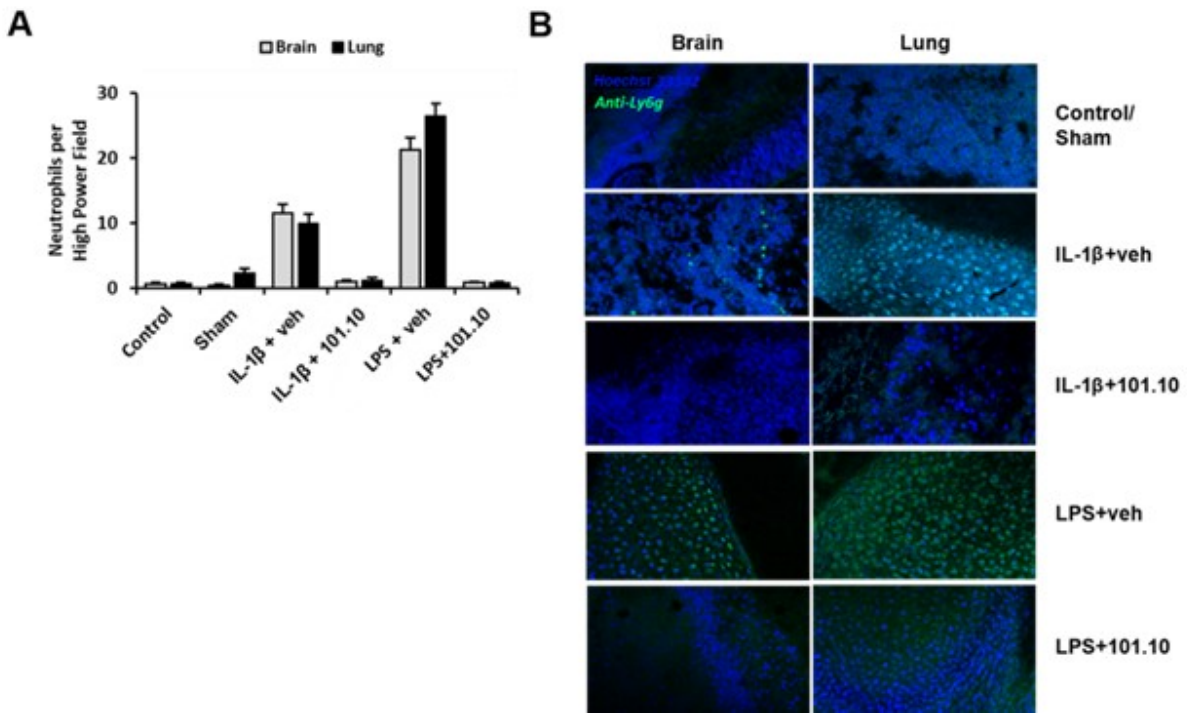

Figure 21. Neutrophil infiltration of the fetal brain and lung in IL-1β- and LPS-induced mouse models of PTB. This effect may be reversible with co-administration of 101.10. Fetuses were collected from mice euthanized at GD17 for the indicated treatment groups (n=2 or 3). Six 7µm tissue sections were prepared from snap-frozen fetal brain and lung and stained for DNA (Hoechst 33342, blue stain) and a neutrophil surface marker (anti-Ly6g, green stain). Four different fields were counted per section under a confocal microscope at 20x magnification, blinded to the specimen details. Areas containing blood vessels and leukocytes within blood vessels were excluded. (**A**) Neutrophil counts in fetal brain and lung tissue for the indicated experimental groups. (**B**) Representative images of neutrophils and their distribution in the fetal brain and lung of the indicated experimental groups. Credit: Meghan Onushko.

yolk sac plays a continuous role through term, and offers a direct route for neutrophils to invade the fetus and instigate fetal inflammation. This would not happen in the human fetal membranes, and may explain why GCF decreases in the murine fetal membranes, as to protect the fetus from an adverse immune attack.

GCF also decreases in the murine cervix as the mouse approaches labour. This is consistent with the absence of further leukocyte infiltration into the cervix after term [7]. As a major of source of neutrophil elastase, collagenase, and MMP9, neutrophils that invade the cervix at term have been hypothesized to help soften the cervix. Once this process is complete, there may be negative feedback to prevent further infiltration of leukocytes, involving a decrease in GCF at the cervix.

Neutrophil Invasion of the Mouse Lower Uterus during IL-1β-Induced PTB. Our data does not support our hypothesis that IL-1β stimulates murine neutrophil invasion via increased GCF. The GCF did not rise with the increase in neutrophil density in the mLU after PTB induction with IL-1β, converse to what we found at during normal pregnancy at GD18.5. Consistent with this finding, IL-1β did not affect the secretion of GCF in hFM, whether TNL or TL. This is surprising given that one of the primary mechanisms of IL-1β-mediated neutrophil recruitment is through the secretion of chemotactic factors at the site of inflammation. For example, in acute inflammatory arthritis models in rabbits, IL-1β has been reported to be responsible for the optimal production of the neutrophil chemokines IL-8 and growth-related oncogene [243]. Although mice do not have an IL-8 ortholog, they produce KC and MIP2 which are related to growth-related chemokines and are produced in response to IL-1β at the site of *Staphylococcus aureus* infection in the skin of mice [244].

One possible explanation for these observations is that that we may have collected the GCF at a stage when it is no longer present in the lower uterus. After all, GCF does fall shortly after it peaks during normal parturition, and a similar system may occur here. Alternatively, this is to say that during the final stages of neutrophil recruitment, negative feedback may result in a reduction in GCF to prevent further infiltration of neutrophils. This type of behavior has been observed before in response to group B *Streptococcus*-induced neonatal sepsis, in which TLR2-derived IL-10 production has been characterized as a negative regulator of neutrophil recruitment [245]. Excessive neutrophil accumulation can be detrimental to both the mother and baby, as can be seen in fetal inflammatory response syndrome [246]. However, neutrophil-associated inflammatory events are often rapid, robust responses that involve positive amplification, rather than negative feedback [247]. If the goal of neutrophil invasion during pregnancy is to weaken fetal membrane integrity and accelerate uterine activation, positive amplification would be more effective than a negative feedback system.

An alternative explanation for our findings is that intrauterine IL-1β administration at GD16 leads to neutrophil invasion independent of an increase in GCF. From our data, IL-1β in our model likely mediates neutrophil invasion via the enhanced leukocyte chemotaxis phenotype. In the context of the literature, this finding may provide a plausible explanation for why peripheral leukocytes of PTB mothers are primed for chemotaxis in response to a standard chemoattractant compared to their non-laboring counterparts [61]. To my knowledge, this is the first time that the administration of IL-1β has been linked to the enhancement of leukocytes for chemotaxis *in vivo*. Previous studies have linked IL-1β to neutrophil recruitment, but only as the effect of an increase in chemokine secretions [243, 244] or the induction of expression of cellular adhesion molecules in the vascular smooth muscle cells to enhance adhesion [248]. The

enhancement of peripheral leukocytes for chemotaxis may be a novel mechanism by which IL-1β can facilitate neutrophil recruitment to an inflamed tissue. This type of enhancement has not been demonstrated yet for IL-1β in other inflammatory diseases, but another pro-inflammatory cytokine, IL-6, has been demonstrated to be a major force in enriching IL-8 mediated migration of $Foxp3^{+}$ CD4 regulatory T cells to IL-8 producing tumors by inducing CXCR1 expression [249]. It may be that IL-1β is similarly inducing the expression of chemokine receptors to enhance responsiveness towards the GCF. In addition, peripheral leukocytes from IL-1β-treated mice have a higher abundance of mRNA for IL-1β, IL-6, TNF-α, and CCL2 than those of sham-treated mice, providing yet further support for greater leukocyte bioactivity as a pregnant dam approaches labor.

In all, our study demonstrates that IL-1β leads to an enhanced leukocyte chemotaxis phenotype that is associated with an influx of neutrophils into the gestational tissues. This influx of neutrophils into the mLU is likely responsible for the increased myometrial expression of IL-1β, IL-6, MMP9, and CCL2 that has been previously reported in this model [40]. First, neutrophils are a major source of cytokines. In the human myometrium and cervix, the production of IL-8 and IL-6 have been immunolocalized to the leukocytes within the tissue [214]. Second, neutrophil products and secretions can also stimulate the expression of proinflammatory cytokines in nearby cells. For example, neutrophil elastase can mediate the expression of TNF-α, MIP2, and IL-6 through a TLR4 dependent pathway [250].

A possible research application from our findings is enabling investigators to study neutrophil invasion *in vivo* in the context of sterile PTB using our model. Historically, mouse models of PTB have relied on LPS, heat-killed *Escherichia coli*, or peptidoglycan, infection-associated agents that stimulate an inflammatory response leading to PTB [251]. PTB without

infection is far more prevalent than those with infection, and infectious mouse models of PTB do not accurately model the physiology of sterile PTB [252]. The knowledge that PTB induction via IL-1β administration results in neutrophil invasion provides another resource for investigators to use for elucidating the regulatory role of immune cells in parturition.

Another important finding of this study is that 101.10 can successfully reverse the effects of IL-1β on both PTB and neutrophil invasion. This effect is consistent with the decreased myometrial expression of proinflammatory cytokines reported in this model after 101.10 co-treatment [40]. 101.10 blocks IL1-activated SAPK/c-Jun and Rho-ROCK pathways leading to AP-1 assembly [40]. This suggests that both PTB and neutrophil invasion pathways utilize IL-1β through the effects of activator protein (AP)-1, independent of NF-κB. There is already evidence of AP-1 playing an important role in labour. Recent studies have demonstrated changes in the expression of AP-1 family members in the uterus and fetal membranes during labour [253-255]. Moreover, AP-1 activation alone is sufficient to induce labour in mice, and inhibition of AP-1 alone is sufficient to delay IL-1β-mediated PTB in mice [39, 40]. The role that AP-1 plays in the regulation of parturition is unclear, but it is known that many proinflammatory genes have AP-1 binding sites, including PGHS2, IL-6, IL-8, and CCL2 [256-258]. In addition, AP-1 is a key regulator of OT receptors, MMP, and CX43 [259-261].

TL and TNL Maternal Serum Factors Enhance Peripheral Leukocytes for Chemotaxis in Response to TL FM GCF. Moreover, our data demonstrate that TL serum can enhance TNL hL chemotaxis in response to TL hFM GCF but not in response to TNL hFM GCF. This outcome suggests that 1) there is some sort of priming factor that is present in the maternal serum that can enhance leukocytes for chemotaxis either by binding to the leukocytes or being taken up by

leukocytes, and 2) there may be a specific receptor that is on the enhanced leukocyte's cell membrane that responds to a factor that is present in TL hFM GCF but not TNL hFM GCF. An alternative explanation for 2) is that there could be a lack of statistical power when comparing low values with limited replications, or that sufficient GCF may be required to provide enough resolution for identifying statistically significant differences between leukocyte activities.

The data also show that TNL serum can enhance TNL hL chemotaxis in response to TL hFM GCF, which was unexpected because one might expect the priming agent to be present in TL but not TNL serum since leukocytes are more active at TL than TNL [4]. It is possible that the incubation process itself could activate leukocytes, but this is unlikely because incubation with neither TL nor TNL hFM GCF enhanced TNL hL chemotaxis in response to IL-8. The priming agent is therefore likely to be present in TNL serum and may exert gradual effects on peripheral leukocytes *in vivo* even if the mother is not yet ready for labour. Further study is needed to determine the exact point in gestation when the priming agent appears in maternal serum.

To begin the process of identifying the priming agent, we screened for 40 different cytokines in TNL and TL maternal serum for future reference. Interestingly, eight cytokines (CCL21, CCL11, IFN-γ, IL-4, IL-6, IL-8, CCL20, and CCL23) increased from TNL to TL but none of the cytokines decreased, even though this screen included both pro-inflammatory (IL-6, IL-1β) and anti-inflammatory (IL-4, IL-10) cytokines. Most of the screened cytokines tended to rise and the eight mentioned above reached significance. A plausible reason for the increase in anti-inflammatory cytokines is to maintain the balance of pro- and anti-inflammatory cytokines to prevent excessive inflammation and subsequent damage to vascular endothelium or tissues. Of interest among these cytokines as the priming agent for enhanced leukocyte chemotaxis, IL-6 is a

likely candidate; pre-incubation of neutrophils with IL-6 (10 or 100 ng/ml) significantly enhances chemotaxis towards IL-8 [60].

Neutrophil Invasion of Fetal Brain and Lung. Before these experiments, it was unknown whether 101.10 could affect neutrophil invasion. There were some clues, considering that the NF-κB pathway is important for selectin expression and neutrophil tethering, and that the Rho-ROCK and SAPK/c-Jun pathways are also important players in neutrophil recruitment. These pathways have been demonstrated to affect chemoattractant secretion, cell polarity, and cell motility [127, 138, 139, 171]. Knowing whether 101.10 affects neutrophil invasion is important not only for a better understanding of how it may act as a therapeutic for PTB, but also for protecting the fetus from fetal inflammation. Our experiments demonstrate that in our IL-1β-induced PTB mouse model, neutrophils not only infiltrate the mLU but also the fetal brain and lung, which could set the stage of fetal inflammatory disease.

We do not know whether these neutrophils are maternal or fetal in origin. If they are maternal, they would have to traverse across one of the three maternal-fetal interfaces at the syncytiotrophoblast, a continuous layer of fused multinucleated trophoblasts which lines the intravillous space and contacts the maternal blood, endovascular trophoblast which lines the maternal spiral arteries and contacts the maternal blood, and the interstitial trophoblast which invades the decidua and contacts mucosal immune cells [262]. One way that maternal neutrophils could get across these interfaces is through syncytial damage: an example of leukocytes entering the fetal compartment is when leukocytes mediate the transport of human immunodeficiency virus to the fetal side [263], and another is when maternal T-cells cross the

placenta to reside in fetal lymph nodes, suppressing fetal antimaternal immunity at least until early adulthood [264].

101.10 has previously been proposed as a PTB therapeutic with the knowledge that it could prolong gestation. There was little evidence, however, that it would improve perinatal outcomes. Several attempts to develop a PTB therapeutic in the past have failed to be brought to market because they lacked evidence of improving perinatal outcomes despite being able to prolong gestation. Our work provides preliminary evidence that 101.10 may be able improve perinatal outcomes. 101.10 could also prevent PTB and leukocyte invasion of the fetal brain and lung in the LPS-induced infectious model of PTB. However, given the low power, more trials must be conducted to gain a more complete understand whether 101.10 can protect the fetus organs from leukocyte invasion, and further study is needed to know whether 101.10 can improve perinatal outcomes. Moreover, the ability of the LMA to detect the difference between GCF from 101.10-treated and 101.10-untreated cases of PTB could suggest another function for the LMA: to confirm whether a batch of 101.10 is active, the LMA could be used to determine whether the 101.10 negates the effects of an intrauterine injection of IL-1β. In other words, the LMA could be used to validate 101.10 activity.

Chapter 4. Interactions between Leukocytes, Fetal Membranes, and IL-1β in Human Parturition.

4.1. Introduction. Parturition is an orchestrated phenomenon that involves complex interactions between different tissues. Recently, a PhD student from our lab, Dr. Kelycia Leimert, characterized the crosstalk between the maternal and fetal intrauterine tissues that results in pro-inflammatory amplification and uterine transition. She demonstrated in a novel co-culture model that merely bathing myometrial cells and fetal membrane explants in shared media for 24 hours causes them to have a vastly different mRNA profile, including an increased abundance of COX2 and IL-6 mRNA (Leimert and Olson, unpublished). In a similar way, the gestational tissues and the invading leukocytes may communicate as it has been demonstrated in other inflammatory conditions: for example, endothelial cells have been demonstrated to provide critical signals like Csf1 for the selective growth and differentiation of macrophages, which become capable of accelerating angiogenesis, promoting tumor growth, and adhere tightly with endothelial cells *in vivo* [213].

Dr. Leimert's work also demonstrates that IL-1β pretreatment of hFM explants increases mRNA abundance for various pro-inflammatory cytokines such as IL-6 and IL-1β (Leimert and Olson, unpublished). The human fetal membranes (hFM) consists of chorion and amnion layers that envelop and protect the fetus throughout pregnancy. As pregnancy approaches termination, the hFM is invaded by leukocytes and demonstrates increased levels of gestational chemotactic factor (GCF) [4]. Guinea pig fetal membranes also demonstrate increased GCF at term labour (TL) compared to term but not-in-labor (TNL) [5]. My initial thought was that IL-1β might stimulate the secretion of GCF, but our findings from Chapter 3 suggest that this is not the case.

With relation to leukocyte invasion, the fetal membranes are a major site for leukocyte invasion during parturition in humans [4], and crosstalk between the leukocytes and the fetal membranes may further promote leukocyte invasion and inflammation of the gestational tissues. IL-1β in the fetal membranes has been immunolocalized to the leukocytes, and many other cytokines including IL-8 and IL-6 have been immunolocalized to the leukocytes in myometrium and cervix suggesting that leukocytes are a major source of cytokines and chemokines in the fetal membranes [214]. It is likely that the fetal membranes can amplify this response because Dr. Leimert's work describes the capacity of the fetal membranes to secrete its own cytokines, and even moreso when stimulated with IL-1β (Leimert and Olson, unpublished).

I have collaborated with Dr. Leimert to understand the crosstalk between leukocytes, fetal membranes, and IL-1β, and how they might contribute to leukocyte invasion.

Rationale, Hypothesis, Experimental Approach. A better understanding of the interactions between leukocytes, fetal membranes and IL-1β may help to elucidate the origins of the priming factor that is present in the maternal circulation as described in Chapter 3. We may also gain some insight into the complex processes involved in amplification of the parturition-associated inflammatory event and the mechanisms by which IL-1β acts to ultimately mediate leukocyte invasion and parturition. This may help to clarify the importance of GCF and enhanced leukocyte chemotaxis as regulatory mechanism of leukocyte invasion, and to better understand whether these effects are associated with IL-1β.

This study focused initially on identifying whether TL or TNL hFM explants, either pre-treated or not treated with IL-1β, produced a priming agent that could enhance TNL leukocyte chemotaxis. Given our findings in Chapter 3 regarding the effect of IL-1β administration on

enhancing leukocyte chemotaxis in mice, we hypothesized that TL hFM explant secretions would be able to enhance TNL leukocyte chemotaxis, and moreso for the IL-1β-pretreated TL hFM explants. We also hypothesized that TNL hFM explant secretions would not be able to enhance TNL leukocyte chemotaxis, but that TNL hFM pre-treated with IL-1β would gain the ability to secrete a priming factor that could enhance TNL leukocyte chemotaxis.

The second objective of this study was to compare the chemotactic factors secreted by TL and TNL hFM explants. Although TL hFM homogenates have better chemotactic activity than TNL hFM [2], we wanted to assess how much of the chemotactic factors come from active secretion by the hFM. We hypothesized that TL hFM explant secretions would stimulate more leukocyte chemotaxis than TNL hFM explant secretions.

Third, we wanted to test whether hFM explant secretions can stimulate leukocytes to produce chemoattractants. We hypothesized that TL hFM explants would stimulate leukocytes to produce chemoattractants and that TNL hFM explants would not. We also expected IL-1β to not have any effect given our findings in Chapter 3 regarding IL-1β and GCF secretions.

4.2. Methods. *Isolation and Treatment of Fetal Membrane Explants*. Intact placentas were collected with consent from TNL women undergoing an elective caesarean section (Royal Alexandra Hospital, Edmonton, AB). hFM explants were excised using a 6 mm tissue punch and washed with HBSS. Explants were plated in a 12-well transwell plate with the chorion facing down. Transwells were filled with DMEM F-12 (HyClone, UT, USA) containing 15% fetal bovine serum (FBS) and 1x antibiotic/antimycotic. Explants were acclimatized for 48 hours at 37^{o}C and 5% CO_2. Treatment solutions of 1 ng/mL IL-1β (MilliporeSigma, Etobicoke, ON, Canada) in DMEM were administered over a 6-hour incubation period. The conditioned medium

was collected and centrifuged to remove cellular debris, and will be referred to as ‘single conditioned medium’.

Leukocyte Incubation with FM Conditioned Medium. TL human leukocytes (hL) were incubated with single conditioned medium for 1 hour at room temperature (RT) under gentle agitation using a stir bar to prevent clumping. 1 hour was selected because it was the longest length of time that TL hL could be incubated with the hFM conditioned medium without clumping. The solution was centrifuged, and the supernatant was collected for use as the chemoattractant in an LMA to assess hL secretions. These secretions will be referred to as ‘double conditioned medium’. Furthermore, the pelleted TL hL were resuspended in RPMI, and their responsiveness to GCF was assessed using an LMA.

4.3. Results. *TL hFM Produces a Priming Agent that Enhances TNL hL Chemotaxis.* TL and TNL hFM explants were pretreated with IL-1β or vehicle for 6 hours, after which the single conditioned media were collected (Fig. 22). These media were incubated with TNL hL for 1 hour, after which the leukocytes were washed and resuspended in RPMI for comparison using an LMA. Incubation of TNL hL with TL hFM secretions at RT increased the responsiveness of the leukocytes to a standard chemoattractant (Fig. 23). Pretreatment of TL hFM with IL-1β did not further enhance TNL hL chemotaxis.

IL-1β-Stimulated TL hFM Elicits Leukocytes to Produce GCF. We compared the GCFs in the single conditioned media (hFM secretions after a 6-hour treatment with IL-1β or vehicle) using an LMA with TL hL. There was no significant difference in TL hL chemotaxis between these

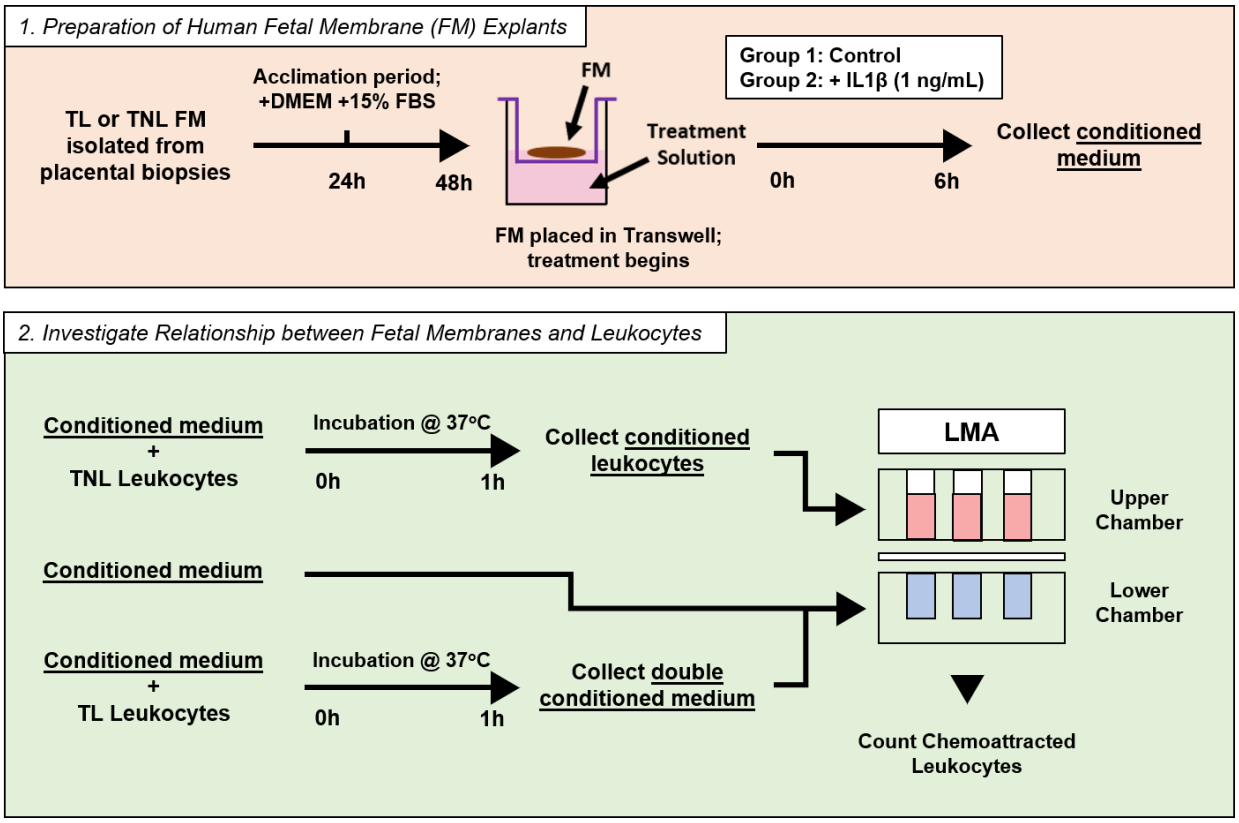

Figure 22. Schematic for culturing human fetal membrane explants and assessing leukocyte response to fetal membrane secretions *in vitro*. Human fetal membrane explants were excised from intact placentas collected with consent from TNL women undergoing an elective caesarean section (Royal Alexandra Hospital, Edmonton, AB). Explants were pretreated with IL-1β, and the supernatant (single-conditioned media) was collected. TL leukocytes were incubated with the supernatant for 1 hour at 37ºC with 5% CO_2, and the resulting supernatant (double-conditioned media) was assessed in an LMA. The single- and double- conditioned media were then tested for chemotactic activity.

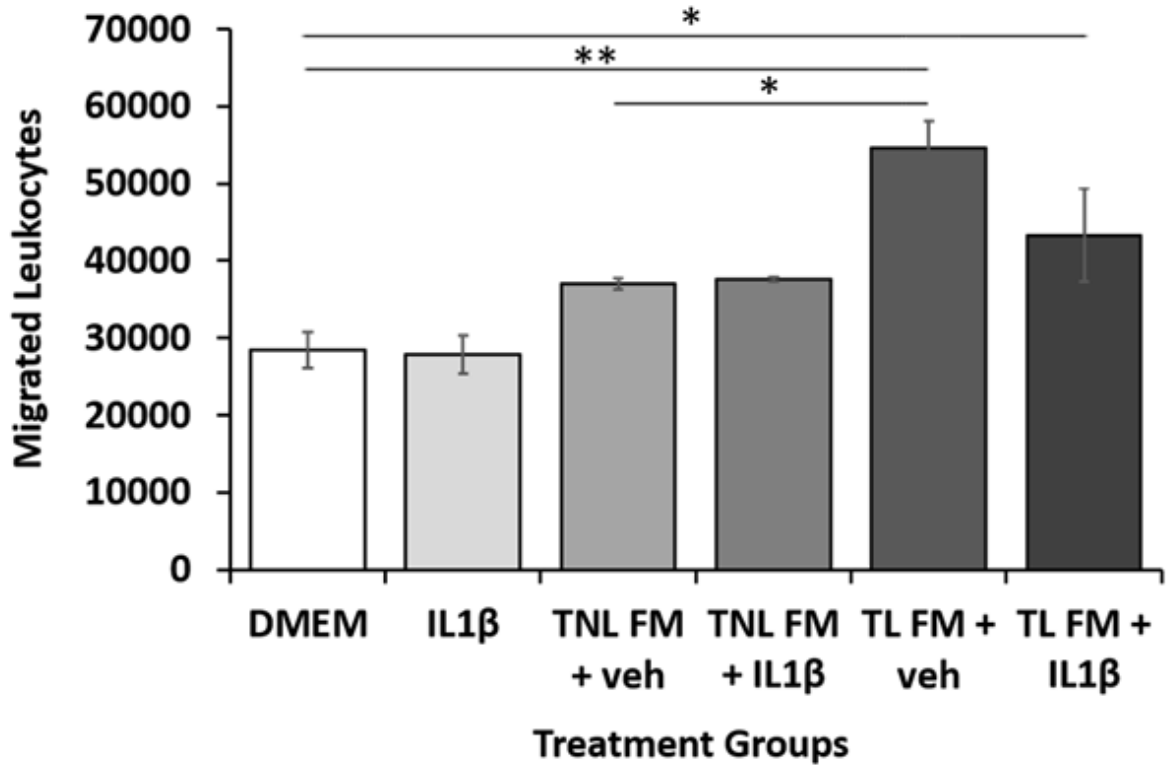

Figure 23. TL hFM explants produce a priming agent that potentiates TNL leukocyte chemotaxis. TNL hL were incubated with hFM explant-conditioned media for 1 hour at RT under gentle agitation to prevent clumping, and then isolated via density centrifugation. An LMA was used to assess the responsiveness of the conditioned leukocytes to TL hFM GCF. Statistical significance was performed using one-way ANOVA ($p<0.001$), and Tukey's HSD test as a *post hoc* test. *$p<0.05$, **$p<0.01$

groups (Fig. 24). We then incubated the single conditioned media with TL hL, collected the double conditioned media, and compared the GCFs in the double conditioned media using an LMA with TL hL. The result was that the double conditioned medium of leukocytes incubated with IL-1β-stimulated TL hFM was more potent than the double conditioned medium of leukocytes incubated with non-treated TL hFM or than basal leukocyte secretions alone (Fig. 25).

4.4. Discussion. We report that enhanced leukocyte chemotaxis can be artificially induced *in vitro* via incubation with TL hFM secretions, which suggests that a priming agent may be secreted by the hFM. Interestingly, IL-1β pretreatment of TL hFM had no effect on the secretion of a priming agent, perhaps because the TL hFM is already doing so at maximal output. To confirm this theory, we could measure the effect of anti-IL-1β on the production of priming agent by TL hFM.

In addition, IL-1β pretreatment of TNL hFM did not stimulate the secretion of a priming agent, but it is unclear whether this outcome is because IL-1β treatment alone is insufficient *in vitro* to stimulate the secretion of a priming agent or because the hFM is not an IL-1β-inducible source of priming agent. What our findings demonstrate, however, is that the hFM becomes capable of secreting a priming agent at TL. Previous work from our lab demonstrates that the TL hFM, when in the presence of other TL gestational tissues, secretes several cytokines at high output (Leimert and Olson, unpublished). A comparison of the changes from TNL to TL hFM has also been conducted by our lab (Yin and Olson, unpublished) that demonstrates an increase in key cytokines including IL-6, which as described earlier in Chapter 3 is a strong candidate for being our priming factor of interest.

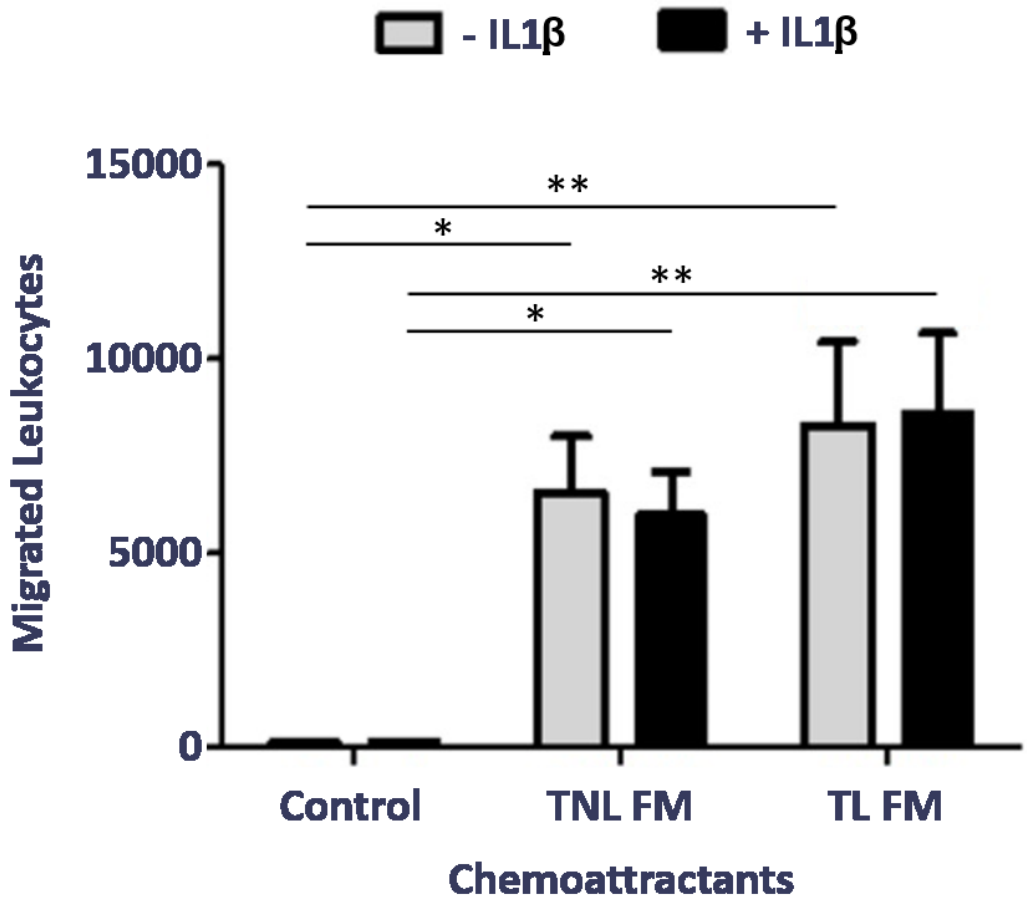

Figure 24. No effect of IL-1β pretreatment on CF production in hFM explants. Single conditioned medium was collected as described in fig 22 (TNL: n=6, TL: n=6) and used in a chemotaxis assay was performed using untreated TL human leukocytes to assess the relative strength of CF. Statistical analysis was performed by two-way ANOVA, and Tukey's HSD test as a *post hoc* test. *$p<0.05$, **$p<0.01$.

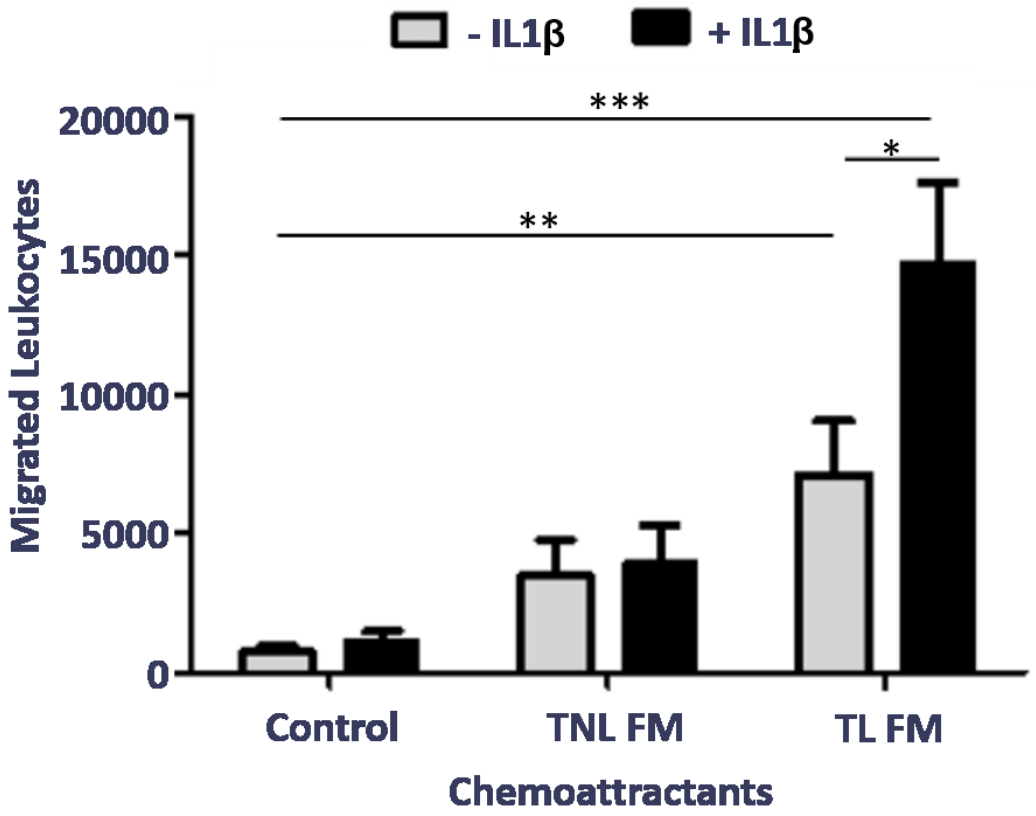

Figure 25. IL-1β stimulates TL hFM to produce an intermediary factor that elicits leukocytes to secrete CF. Single conditioned medium was collected as described in fig 22 (TNL: n=6, TL: n=6). TL hL were incubated with the single conditioned medium for 1 hour, after which the resulting double conditioned medium was collected. A chemotaxis assay was performed using untreated hL to assess the relative strength of CF in the single and double conditioned mediums. Hoechst 33342 was used to quantify the migrated leukocytes. Statistical analysis was performed by two-way ANOVA, and Tukey's HSD test as a *post hoc* test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Chapter 5. General Discussion.

5.1. Main Findings. *Leukocyte Migration Assay Improvements.* The advances described in this thesis have brought us one step closer to developing a diagnostic for PTB. Switching to a 96-well chemotaxis chamber with larger individual wells has helped us resolve the chemotaxis step twice as fast as with the 48-well chemotaxis chamber, and using fluorescence spectroscopy to quantify total migrated leukocytes is a faster, cheaper, and more precise technique than flow cytometry. Overall, the LMA improvements led to a >60% faster assay that costed >80% cheaper.

Leukocyte Invasion and the IL-1β-induced PTB Mouse Model. To our knowledge, this is the first report that IL-1β-induced PTB in mice is associated with leukocyte infiltration of the lower uterus (mLU). This finding also means that this model is the first non-infectious model of preterm birth to be associated with leukocyte invasion. As such, the current study is valuable in that it provides researchers with a model that can be manipulated to better understand the immunological control of PTB. Moreover, exploring this model could lead to new molecular targets for PTB diagnosis and therapy.

During our study, we confirmed that a single intrauterine injection of IL-1β in the space between two fetal sacs elicits PTB in CD1 mice. We also confirmed previous findings that IL-1β administration also results in peripheral leukocytes that have a greater abundance of mRNA for the proinflammatory cytokines IL-1β, IL-6, TNF-α and CCL2 with similar degrees of change [144]. Our findings also validate that the model is replicable across laboratories.

Leukocyte Invasion Characteristics in Mice. With regards to characterizing the timing and targeting of leukocyte invasion during normal parturition in mice, we identified that of the five

tissues we tested, namely lower uterus, upper uterus, fetal membranes, placenta, and cervix, the mLU was the only one that experienced a significant increase of GCF towards labor, suggesting that the mLU is the primary target for leukocyte invasion. A comparison of GCF in the mLU throughout gestation demonstrated that GCF peaks at GD18.5, just before labor onset, and falls back to normal levels one day post-partum. This is similar to previous findings that total GCF in human fetal membranes (hFM) rises from both preterm and term not in labor to the onset of spontaneous term labor [56, 238]. Moreover, in guinea pigs and rats, the secretion of GCFs in amniochorion and uterus increase throughout late gestation respectively [3, 5].

Leukocyte Invasion in Normal Parturition vs PTB. With regards to comparing the regulation of leukocyte invasion during normal parturition and IL-1β-induced PTB in mice, we identified that whereas both an increase in GCF at the mLU, and an enhanced ability of leukocyte to migrate to these GCF are associated with leukocyte infiltration of the mLU in normal parturition, only enhanced leukocyte chemotaxis was associated with leukocyte infiltration of the mLU in our PTB model. There are at least two significant outcomes of these findings:

First, the fact that mLU does not have more GCF even though we detect greater neutrophil infiltration suggests that the targeting of the mLU is achieved by another means. This could be accomplished by the IL-1β-induced expression of cellular adhesion molecules in the vascular smooth muscle cells (VSMCs) to enhance adhesion of leukocytes [248], as well as by the IL-1β-induced production of TNF-α [265] which promotes vasoconstriction of postcapillary venules via stimulation of ET-1 production and release [266]. Vasoconstriction can lead to reduced blood flow and greater opportunity for leukocytes to contact the adhesion molecules on VSMCs. Greater leukocyte adhesion to the VSMC at the target site then allows for leukocytes to

be more responsive to chemotactic signals at the site of inflammation, which in this case is the mLU.

Second, we have demonstrated that IL-1β administration in mice can result in changes to the phenotype of circulating leukocytes that enhance their ability to undergo chemotaxis. This is a novel finding, and it is unknown whether IL-1β facilitates such an effect only during pregnancy or whether this is a basic physiological phenomenon. The first thought might be that IL-1β stimulates the expression of chemokine receptors directly as it does for CXCR4 in Tongue Squamous Cell Carcinomas [267]. Another example of this type of enhancement has been demonstrated for IL-6, which is a major force in enriching IL-8 mediated migration of $Foxp3^{+}$ CD4 regulatory T cells to IL-8 producing tumors by inducing CXCR1 expression [249]. However, our results in Chapter 4 (Fig 19) clearly demonstrate that IL-1β does not directly stimulate enhanced leukocyte chemotaxis. Rather, our data suggest that there is an intermediary priming factor(s) that can be produced by TL hFM explants.

Although this priming factor is unknown, our lab previous analyzed the cytokines secreted by TL hFM and demonstrated that they were a major source of pro-inflammatory cytokines (IL-6, CCL2, TNF-α) (Leimert and Olson, unpublished). Moreover, my data demonstrate that maternal serum contains a priming factor that can enhance TNL leukocytes for chemotaxis in response TL hFM GCF. Whether this serum priming factor is the same as the one secreted by TL hFM is unknown, but a screening analysis of maternal serum cytokines demonstrated that TL maternal serum contains higher levels of CCL21, CCL11, IFN-γ, IL-4, IL-6, IL-8, CCL20, and CCL23 than TNL maternal serum. Comparing the cytokines in both TL hFM secretions and the TL maternal serum, one that stands out as a priming factor candidate is

IL-6 because pre-incubation of neutrophils with IL-6 (10 or 100 ng/ml) significantly enhances chemotaxis towards IL-8 [60].

101.10, a Preterm Birth Therapeutic in Development. This thesis demonstrates that both leukocyte invasion of the maternal uterus and PTB in mice can also be blocked by 101.10. Leukocyte invasion can potentially be harmful to the fetus during PTB, as suggested by recent reports that IL-1β-induction of PTB negatively impacts the fetus [140]. These detrimental effects could be an unintended consequence of leukocyte invasion: leukocyte secretions are pro-inflammatory and may result in chorioamnionitis when leukocytes are present at high densities [268]. Chorioamnionitis is characterized by the presentation of feverish symptoms (>38^{o}C or >100.4^{o}F) and at least two other signs (uterine tenderness, maternal or fetal tachycardia, and pungent amniotic fluid), and it is a common complication of pregnancy that is associated with a plethora of adverse maternal outcomes including endometriosis and postpartum hemorrhage, and adverse perinatal outcomes including perinatal death and septic shock [269]. In preventing leukocyte invasion, 101.10 could therefore also protect both the mother and fetus from adverse inflammatory injury as well as preventing PTB.

We also demonstrated early evidence that 101.10 can protect the fetus from inflammatory disease by preventing the infiltration of neutrophils into the fetal brain and lung tissues in our IL-1β-induced PTB mouse model. More replications of the study will be necessary to affirm this statement. Furthermore, we have early evidence that 101.10 can prevent neutrophil infiltration of the fetal brain and lung in an infectious mouse model of LPS-induced PTB. This finding is interesting from a basic science and clinical science perspective. From a basic science view, it demonstrates that 101.10 antagonism of IL1R1-mediated AP-1 effects is sufficient to prevent

LPS-mediated neutrophil invasion of these fetal tissues. LPS is a well-known TLR4 agonist that can stimulate the splicing and translation of stored pro-IL-1β RNA [270]. As such, LPS may mediate neutrophil invasion via an IL-1β- and AP-1-dependent mechanism that can be prevented by 101.10. From a clinical perspective, 101.10 may be able to fill the void left by an absence of PTB therapeutics. Our data demonstrate that 101.10 is a promising new therapeutic with early evidence of not only being able to prevent PTB, but also to improve neonatal outcomes.

5.2. Leukocyte Invasion in an Updated Model of Parturition. Overall, we have advanced our understanding of how leukocyte invasion may be being regulated before labor onset in normal pregnancy and preterm birth, and our knowledge of the role of IL-1β in parturition (Fig. 26).

On the maternal side of the model, DAMPs and PAMPs activate toll-like receptors to initiate the parturition cascade with the production of the key cytokine, IL-1β. An alternative way for an increase in IL-1β to occur is through the functional withdrawal of progesterone theory that has been proposed to be a key player in the hormonal control of parturition: the shift from low to high PR-A:PR-B results in increased IL-1β [62]. We have demonstrated that this IL-1β can then stimulate enhanced leukocyte chemotaxis indirectly. We have shown that an intermediary priming factor can be secreted by TL hFM and is present in TL maternal serum that can enhance leukocyte to migrate towards GCF. Simultaneously, pro-inflammatory cytokines are locally produced at the gestational tissues, and the crosstalk between the various gestational tissues likely results in synergy and further amplification of the inflammatory response. Our data also suggests that as a pregnancy approaches termination in humans, the fetal membranes, stimulated by IL-1β, secrete an intermediary factor that can stimulate leukocytes to secrete chemotactic

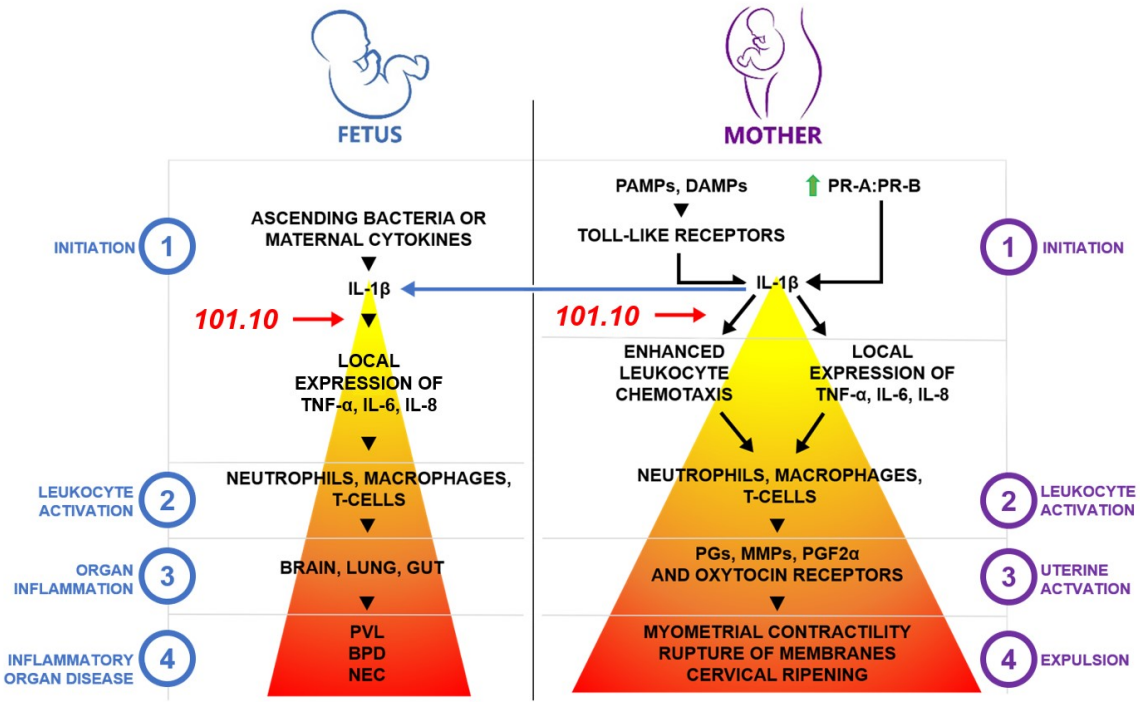

Figure 26. Our Updated Model of Parturition. The current study demonstrates that IL-1β elicits preterm birth, neutrophil invasion, and enhanced leukocyte chemotaxis. We demonstrated that these events can be blocked by co-administering rytvela. As well, the current study suggests that there is a maternal transmission of cytokines to the fetus which leads to neutrophil infiltration of the fetal brain and lung. Rytvela was able to prevent this effect. Since neutrophil infiltration of the fetal organs can lead to fetal inflammatory diseases, rytvela may be important as a protective agent for fetal inflammation.

factors. This process may help to further recruit leukocytes to these tissues. Once leukocytes are recruited, the remainder of the model is largely unchanged.

However, an additional finding from our study is that following IL-1β administration in mice, there is a transfer of inflammation to the fetus across the placenta that results in the infiltration of neutrophils into the fetal brain and lung. This is important because fetal brain injury, which is a major cause of infant mortality and childhood disability, often begins *in utero* with inflammation [271]. The presence of neutrophils, microglia, and macrophages in the fetal brain have been associated with chorioamnionitis and injury throughout the placental-fetal-brain axis [271]. Moreover, chorioamnionitis has been associated with severe infiltration of macrophages, neutrophils and lymphocytes to the human fetal lung [272]. This phenotype of leukocyte invasion is similar to what is found during acute respiratory distress syndrome, and the cytokines these leukocytes release in the fetal lungs could contribute to the pathogenesis of acute and chronic lung disease [272]. Where these neutrophils come from (maternal or fetal) and how they reach these fetal tissues (especially through the placental barrier if these leukocytes are maternal) is unknown.

5.3. Future Directions. The knowledge that we have gained from these studies has only left us with even more questions and, by extension, opportunities for exploring the mechanisms of parturition and the development of PTB diagnostics and therapeutics.

Bringing the LMA to Clinical Practice. Even though several improvements were made to the LMA in this thesis, much work is still needed to bring the LMA to the clinic. Most importantly, a better clinical implementation strategy is necessary. Due to limitations with sample acquisition, the ROC curve generated for the current iteration of the LMA as published by Takeda *et al* [4] is

only able to predict labor within 7 days if the woman delivers at a specific gestational length (39w+0) rather than a range of days.

Therefore, an important step for the development of the LMA might be to conduct a prospective study seeking to generating ROC curves based on the length between the time at which the clinical diagnostic is performed and the time at which labor occurs. More specifically, pregnant women could be recruited and their whole blood could be collected at TNL when they come in for a final check-up around a week before labor is expected. These patients could be tracked until their onset of labor, when another blood sample would be collected. The TNL data can be grouped by the number of days it takes for the corresponding patient to undergo labor at TL (10d TNL, 9d TNL, 8d TNL, etc…). Each of the TNL groups can be compared to the TL data to generate an ROC curve for the prediction of labour onset within a corresponding number of days. A cut-off can then be calculated for the number of migrated leukocytes that demonstrates the best sensitivity and specificity for each TNL group. Using these cut-offs, a secondary study could be done to determine the accuracy of the assay during normal parturition, and a tertiary study could be done to determine whether this assay can predict preterm labor.

Another important step for the development of the LMA is to design a chamber that can accomplish the same task as the current chemotaxis chamber but can test a single patient. For example, a 3-well chemotaxis chamber that uses the same dimensions and possesses the same physical qualities as the 96-well chemotaxis chamber would be effective. In collaboration with the Engineering Garage in the Faculty of Engineering (University of Alberta), I have printed a very simple 3-well chemotaxis chamber made of PolyJet materials using the Stratasys J750 3D printer (Stratasys Ltd, Eden Prairie, Minnesota, USA) that is a promising prototype. It is water

resistant and retains liquid in the top and bottom wells for over 2 hours at 37ºC, and it is cheap and disposable at $2 per chamber.

Yet another step that could be crucial to the development of the LMA is to determine an optimal mix of recombinant chemokines to use as the chemoattractant. The original LMA uses a pool of TL human fetal membrane homogenates, which is impractical for clinical implementation. Beyond the ethical concerns regarding the collection of human tissues for monetary purposes, these homogenates are limited in quantity, and one batch of homogenates will likely be different from another. As such, using a combination of standard chemoattractants in the LMA will be more feasible and reliable going forward. Synergy between chemokines has already been demonstrated for stimulating neutrophil migration [112]. Specifically, the chemotaxis of neutrophils in response to 5 ng/mL IL-8 was much greater in the presence of 100 or 300 ng/mL MCP1. The ideal concentrations of IL-8 and MCP1 for eliciting leukocyte chemotaxis are unknown, and future studies can determine these concentrations for application in the LMA.

Trigger for Leukocyte Invasion and Parturition. With regards to the regulation of leukocyte invasion and how it might be involved in the timing of parturition, it is curious how the initial surge of IL-1β that mediates leukocyte invasion occurs. As discussed in Chapter 1, the current theory is that a functional withdrawal of progesterone regulates the timing of parturition in humans. It has been suggested that this functional progesterone withdrawal is associated with an increase in the expression of IL-1β at the maternal uterus. Therefore, this functional withdrawal of progesterone may initiate an IL-1β-mediated recruitment of leukocytes by enhanced leukocyte chemotaxis. Future studies could look at leukocyte invasion and enhanced leukocyte chemotaxis

in a model of artificially-induced functional progesterone withdrawal. Although this model may not exist yet, it might be developed in the future. In mice, systemic progesterone withdrawal and its effect on leukocyte invasion may also be characterized by inducing luteolysis by artificially activating COX-1 or by administering $PGF_{2\alpha}$.

Further Characterization of Leukocyte Invasion. In our study, we detected neutrophils at the mLU and fetal brain and lung because our lab previously identified them as the primary group that underwent chemotaxis in response to GCF in an LMA (Yin and Olson, unpublished). Furthermore, it was the primary population of migrated cells reported in our publication [4]. However, it is still worth probing for the other subpopulations of leukocytes, such as macrophages, T-lymphocytes, and mast cells, that might infiltrate the mLU in our IL-1β-induced mouse model of PTB.

Targeting of Leukocyte Invasion. My second question is how leukocytes are trafficked to the gestational tissues during parturition or to the lower uterus in our IL-1β-induced mouse model of PTB. Our initial hypothesis that this targeting of the gestational tissues was mediated by an increase in GCF was incorrect. As described earlier, an alternative pathway could involve the upregulation of endothelial CAMs at the uterine arteries to facilitate diapedesis. Future studies could determine whether targeting occurs in this way by first comparing the density of leukocyte in the uterus and the other systemic tissues, and then comparing the mRNA abundance for endothelial CAMs at the uterine arteries in PTB- and non-induced mice. Additionally, IL-1β-mediated vasoconstriction via stimulation of TNF-α and endothelin-1 production could also help

slow the flow of blood at the gestational tissues to increase the opportunity for leukocytes to transiently bind vascular smooth muscle cells and undergo diapedesis.

Identification of the Gestational Chemotactic Factor(s). Even though GCF does not increase in our PTB model, it may still play an important role in normal parturition, and I am curious as to what the identity of the GCF is. It may be one compound or a composite of many compounds that work synergistically as seen with IL-8 and CCL2. Knowing what it, however, is could lead to a better understanding of leukocyte invasion as well as a method of predicting when normal parturition will occur. While predicting normal parturition may not be medically groundbreaking, mothers may feel more at ease if they knew exactly when they were going to enter normal labour. To this end, future studies may screen for known cytokines in the mLU homogenate as a starting point for identification of the GCF. Separatory techniques such as size exclusion chromatography may be used to separate the homogenate into fractions of different sized proteins, and the LMA can be used to identify the functional fraction.

Characterize "Enhanced Leukocyte Chemotaxis". Even though leukocytes in our IL-1β-induced model of PTB are more chemotactically active compared to control, not much is known about how this phenotype is evoked. One possible theory is that there are more chemokine receptors on these leukocytes for the GCF, and future studies could measure mRNA abundance for the various chemokine receptors in these leukocytes. Another theory is that they can better generate a secondary gradient of chemotactic factors (ie. LTB_4 contained in exosomes [275]) that amplifies the effect of the primary chemotactic factor. A way to test this theory is to measure the amount of LTB_4 released by neutrophils that are exposed to GCF.

Moreover, it would be interesting to see whether this enhancement is a parturition specific phenomenon or whether it occurs during other inflammatory conditions. For example, IL-1β induced pathological conditions in animal models could be studied to see whether their peripheral leukocytes also demonstrate an enhanced ability to undergo chemotaxis.

Identification and Characterization of the Intermediary Priming Factor(s). Our study suggests that there is a priming factor that can enhance leukocyte chemotaxis in peripheral circulation and is present in maternal serum. To identify the priming factor(s), antibodies for candidate factors, like IL-6, can be added to the maternal serum before leukocytes are conditioned by it. Antibodies for the priming factor(s) should diminish the enhancing effect on leukocytes. A similar loss-of-function study can be done with TL hFM explant secretions because our study suggests that TL hFM explants also secrete a priming factor.

Furthermore, when the priming factor is present in maternal circulation is unknown. Our study shows that priming factor is present at TNL, so it likely presents itself earlier in gestation. Future studies can test whether PTNL samples can enhance leukocyte chemotactic activity.

Leukocyte Invasion of the Fetal Brain and Lung. Further replications of the study are necessary to affirm that leukocyte invasion of the fetal organs occurs in our IL-1β- and LPS-induced mouse models of PTB and that 101.10 can block this effect. If it is confirmed, it would be interesting to determine whether they are maternal or fetal in origin.

101.10 Development. Finally, the development of 101.10 as a PTB therapeutic is another direction for future work. Currently, our lab is planning to collaborate with a lab in China to test

whether 101.10 can prevent PTB in the non-human primate, a necessary leap towards bringing 101.10 to the clinical testing phase of its development. In the future, focus will be given to understanding its effectiveness in animal models and using it to better understand the role of IL-1β in parturition onset. Studies in the far future may focus on determining a safe dosage for 101.10 in humans as well as assessing its effectiveness in preventing PTB and improving perinatal outcomes.

Chapter 6. Conclusions.

The experiments in this thesis ultimately present a deeper understanding of leukocyte invasion in mouse and human parturition than before. Our findings confirm the work of many others before us that leukocyte invasion is a parturition phenomenon, and that its regulation is dependent on cytokine interactions.

Our work establishes IL-1β as a key mediator of parturition, promoting inflammation by enhancing leukocyte chemotaxis peripherally for migration into the gestational tissues where the leukocytes may mediate pro-labour effects. To our knowledge, we are the first to demonstrate that IL-1β administration in mice to induce PTB is associated with leukocyte invasion of the maternal uterus as well as the fetal brain and lung. We are also the first to show that 101.10 can successfully reverse the effect of IL-1β on leukocyte invasion in the mouse. The data suggest that an unknown priming agent is central to the effects of IL-1β on leukocyte invasion.

We have also made advances in the development of a PTB diagnostic and therapeutic, and hope that one day we will live in a world where all births are safely navigated with positive outcomes for both the mother and the baby.

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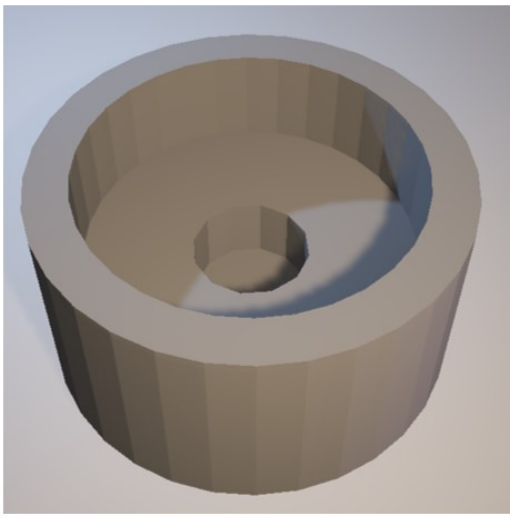
Appendix

Upper Chamber



Lower Chamber

Appendix 1. Preliminary designs for a 3D-printed chemotaxis chamber for the leukocyte migration assay. The upper chamber was meant to be directly printed onto a polycarbonate filter with 3 μm pores. Upon filling the smaller well of the lower chamber with chemoattractant, the upper chamber could be fit into the lower chamber. The chamber could be frozen in this state until use, when leukocytes could be loaded into the upper well. After migration, the leukocytes that have migrated through the filter into the bottom chamber could then be collected for further analysis.

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