INFLAMMASOME ACTIVATED IL-1β AND THE SWITCH TO PATHOLOGICAL INFLAMMATION IN THE BOVINE ENDOMETRIUM

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Doctor of Philosophy (Ph.D.)

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DECLARATION

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work. I agree to deposit this thesis in the University’s open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

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February 2019
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PUBLICATIONS

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ABBREVIATIONS

°C  Degrees Celsius
µm  Micron
AI  Artificial insemination
AIM 2  Absent in melanoma 2
AMP  Antimicrobial peptide
ANOVA  Analysis of variance
APP  Acute phase protein
ASC  Apoptosis associated speck-like protein containing a CARD
ATCC  American Type Culture Collection
ATP  Adenosine triphosphate
BCA  Bicinchoninic acid assay
BEND  Bovine endometrial epithelial cell
BLAST  Basic Local Alignment Search Tool
BoHV4  Bovine herpes virus 4
Bp  Base-pair
BSA  Bovine serum albumin
CARD  Caspase activation and recruitment domain
CD  Cluster of Differentiation
cDNA  Complementary deoxyribonucleic acid
CE  Cytological endometritis
CO₂  Carbon dioxide
CVM  Cervico-vaginal mucus
DAB  3,3'-Diaminobenzidine
DAMP  Danger associated molecular pattern
DC  Dendritic cell
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
DPP  Days post-partum
E. coli  Escherichia coli
EAE  Experimental Autoimmune Encephalomyelitis
EDTA  Ethylenediaminetetraacetic acid
ELISA  Enzyme linked immunosorbent assay
FBS  Fetal Bovine Serum
FITC  Fluorescein isothiocyanate
FRT  Female reproductive tract
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
H&E  Haematoxylin and eosin
H3F3A  H3 histone family member 3A
HBSS  Hanks Balanced Salt Solution
HDP  Host Defence Peptide
Hp  Haptoglobin
Hr  Hour
IFI16  Interferon gamma inducible protein 16
IHC  Immunohistochemistry
IL  Interleukin
ISG  Interferon stimulated gene
ITS-X  Insulin Transferrin Selenium X
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<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TP</td>
<td>Total protein</td>
</tr>
<tr>
<td>UCSC</td>
<td>University of California Santa Cruz</td>
</tr>
<tr>
<td>uNK</td>
<td>Uterine Natural Killer cell</td>
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<tr>
<td>WFDC</td>
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**ABSTRACT**

Inflammation of the postpartum uterus is a normal physiological event, crucial for tissue involution and repair. Immediately following parturition, the uterus experiences dysregulation of its microbiome and is exposed to inflammatory DAMPs as a result of tissue repair mechanisms. Endometrial epithelial cells and underlying stromal fibroblasts provide appropriate innate defence mechanisms to maintain the local microbiome, while also retaining the capacity to respond to pathogens when required. However, in the bovine, some cows fail to resolve this inflammation, resulting in a switch from this physiological inflammatory event to that of a pathological inflammatory phenotype, termed endometritis, which compromises fertility. Previous work from our group identified elevated levels of IL-1β expression in cows with uterine disease (Foley, Chapwanya et al. 2015, Adnane, Chapwanya et al. 2017). We hypothesized that inflammasome mediated cleavage of the inflammatory cytokine IL-1β is a key driver of pathological inflammation in endometritic cows.

We began by establishing a primary culture model of endometrial cell populations. We processed bovine endometrial tissue from non-pregnant bovine uteri immediately post-mortem to obtain primary epithelial cells and stromal fibroblast populations, separated using differential size filtering. Cell transport and isolation conditions were optimised. Following expansion in culture, the purity of cell populations was confirmed using morphology and positive staining for cytokeratin and vimentin which identifies epithelial and stromal fibroblast populations, respectively. Using PCR, cDNA from both cell populations was negative for CD45, a marker of immune cells. On challenge with a bacterial PAMP (LPS), epithelial and stromal fibroblasts showed a marked increase in the expression of the inflammatory mediators IL8, IL6, S100A8 and S100A9, with both cell populations displaying distinct expression profiles.

In order to investigate IL-1β as a possible driver of pathological inflammation, endometrial tissue samples were obtained at 7 and 21 days post-partum (DPP) from cows that developed endometritis at 21 DPP and cows that remained healthy throughout involution. IL-1β was measured by ELISA and immunohistochemistry. Seven DPP, endometrial IL-1β protein levels were significantly higher in animals that proceeded to develop endometritis at 21 DPP with production by the epithelium (both luminal and
glandular), underlying stromal fibroblasts as well as infiltrating immune cells. Stimulation of primary endometrial epithelial cells, stromal fibroblasts and PBMCs with LPS and the inflammasome activator nigericin induced high levels of IL-1β. Stromal fibroblast cells were particularly potent producers of IL-1β. Furthermore, basolateral LPS stimulation of polarized epithelial cells induced IL1B mRNA and a previously undescribed IL-1β protein isoform, with preferential protein secretion into the apical compartment. Stimulation of polarized epithelial cells following a calcium switch assay with IL-1β demonstrated the ability of this cytokine to delay the re-establishment of the epithelial barrier. Key inflammasome components (NLRP3, NEK7, ASC and Gasdermin-D) were expressed by endometrial cells following stimulation. Endometrial cell stimulation in the presence of NLRP3 receptor (MCC950) and pan-caspase (Z-VAD-FMK) inhibitors blocked IL-1β production, demonstrating its dependence on the NLRP3 inflammasome and on caspase activity. Interestingly, in contrast to inflammasome pathways detected in other species, expression of caspase-4 but not caspase-1 was detected. Caspase-4 specific siRNA prevented IL-1β production, confirming that inflammasome activation in endometrial cells is caspase-4 but not caspase-1 dependent.

In conclusion, this study has successfully established a reproducible primary culture model of bovine endometrial cell populations. This culture model was used to demonstrate inflammasome activity in endometrial epithelial cells and stromal fibroblasts. Species- and tissue-specific differences in bovine endometrial cell inflammasome assembly were also revealed. Identifying the mechanisms of inflammasome activation operating in the bovine has critical relevance for our understanding of inflammation and suggests new therapeutic targets for the resolution of inflammatory pathologies in the bovine, and in particular for the prevention of bovine endometritis.
Chapter 1 Introduction

1.1 Inflammation

Inflammation is defined as the host’s protective response to noxious stimuli such as tissue damage or infection. It is an attempt by the host to eliminate infectious or disease causing agents and repair tissue damage in an effort to restore homeostasis (Medzhitov 2008). The cardinal signs of inflammation: calor, dolor, rubor and tumor (heat, pain, redness and swelling), were first described by Celsus in 25 AD (Celsus 25 AD). Since this initial description of the hallmarks of inflammation, we have developed a greater understanding of the mechanisms surrounding an inflammatory event, including the ability of cells to recognise an immunological threat, the subsequent signal transduction pathways which ultimately result in the production or pro-inflammatory cytokines and the recruitment and the effector mechanisms of immune cells drawn to the site of inflammation by the chemokine gradient. There is also a growing awareness that inflammation is a dynamic process which can adapt to suit the threat posed, for example cytokine production and the activation of immune cells are hallmarks of the response to pathogenic stimuli while following physical injury, the activation of the coagulation cascade is crucial to controlling blood loss (Tracy 2006). The process of inflammation has been attributed three main functions; the first to eliminate the cause of the inflammatory insult; secondly, to prevent the spread of infection and finally to initiate mechanisms of tissue repair (Medzhitov 2008).

While initial models of immune recognition and activation focused on the ‘stranger’ model, or detection of non-self, the proposal of the ‘danger’ model which suggests that the immune system is more concerned with the detection of damage than with foreign entities has allowed us to generate a greater understanding of inflammatory processes and the essential requirement to return the host to a state of homeostasis (Janeway 1989, Matzinger 1994). The danger model also accounts for inflammatory events initiated by stressed or malfunctioning cells. Arising from this model is the concept of healthy tissues promoting a state of tolerance in order to maintain homeostasis while distressed tissues evoke an immune response (Matzinger 2002).
The hallmarks of inflammation described by Celsus are a direct result of the increased blood supply to the sight of infection or tissue damage and represent an attempt to coordinate the trafficking of immune cells to the site of injury. Initial recognition of the pathogenic insult is mediated by a host of cell types including the local epithelium, cells comprising the stromal matrix, in addition to local tissue resident immune cell populations such as macrophages. Detection and recognition of either pathogenic organisms or tissue damage is mediated by a wide range of innate immune receptors, comprising the Toll-like receptor (TLR) family, the NOD (nucleotide binding-oligomerisation-domain protein)-like receptor (NLR) family receptors and RIG-I receptor families. These pattern-recognition receptors (PRRs) are capable of detecting a wide range of pathogen associated molecular patterns (PAMPs) such as the bacterial product lipopolysaccharide (LPS), microbial associated molecular patterns (MAMPs) and damage associated molecular patterns (DAMPs) which are indicative of alterations in the homeostatic environment (Barton 2008).

The TLR family of PRRs are essential for the recognition of bacterial, viral and damage associated molecules and play a central role in the initiation of an inflammatory response. The homodimers TLR4, TLR5 and TLR11 and the heterodimers TLR2 and TLR1 or TLR2 and TLR6 are located on the plasma membrane while TLR3, TLR9, TLR13 and the heterodimer of TLR7 and TLR8 are localised to endosomes within the cell in an effort to recognise pathogen or host derived nucleic acids. Engagement of the PRR results in the dimerization of the TLR receptors and initiates the subsequent signalling cascade within the cell. These signalling cascades ultimately converge in the activation of a number of transcription factors, which depending on the PRR pathway engaged, can include nuclear factor κB (NFκB), interferon regulatory factors (IRFs) and activator protein (AP-1). The end result of these signalling pathways is the induction of pro-inflammatory cytokines and in the case of viral sensing through the endosomal TLRs, the induction of type I interferon (O'Neill, Golenbock et al. 2013).

The production of inflammatory mediators such as cytokines and chemokines marks the initiation of the effector phase of the inflammatory response. The activation of the blood vessel endothelium by the secreted inflammatory cytokines allow for selective extravasation of immune cells such as neutrophils (a process known as diapedesis) while restricting erythrocytes from exiting the blood vessels (Pober and Sessa 2007). Once they have exited the blood vessels, the immune cells are immediately recruited to the site of
inflammation by the chemokine gradient. While chemokines such as IL-8 function to recruit immune cells to the site of inflammation, other pro-inflammatory cytokines such as IL-1α, IL-1β and IL-18 (members of the highly potent IL-1 family of cytokines) drive activation and differentiation of immune cells at the sites of inflammation. Their main function is to elicit and restrict an inflammatory event to the local site of infection.

Upon reaching site of inflammation, immune cells become activated by direct contact with pathogens or exposure to DAMPs or activating cytokines produced by the local tissue resident cells. Immune cells belonging to the innate arm of the immune system are first to respond with neutrophils being the first cells recruited to the site of inflammation. These are assisted by tissue resident macrophages and monocytes recruited from the peripheral blood. Dendritic cells provide the link to the adaptive immune system, through the sampling and subsequent presentation of antigen to T cells. Involvement of the adaptive immune response can result in the development of immunological memory, whereby memory T and B cells can recall previous encounter with an antigen and respond by mounting a rapid and potent immune response (Ahmed and Gray 1996). While immune cells have classically been thought to have to be recruited to the site of inflammation, there is a growing awareness of the presence of tissue resident immune cell populations, in particular tissue resident macrophages and NK cells. Tissue resident immune cell populations, distinct from their peripheral blood counterparts, have been described in several unique immunological organs such as the liver and uterus (Critchley, Kelly et al. 2001, Lysakova-Devine and O'Farrelly 2014, Harmon, Robinson et al. 2016).

Following activation at the site of the inflammatory insult, immune cells utilise a number of potent effector mechanisms. These include the release of toxic granules, reactive oxygen species and host defence peptides such as defensins and cathelicidins (Majno and Joris 2004). These inflammatory components are aimed at eliminating the invading pathogenic threat or repairing and remodelling the tissue damage in an effort to return to a state of homeostasis, however these effector molecules are unable to distinguish between the host and the inflammatory threat, meaning tissue damage to the host is inevitable (Nathan 2002).

An acute inflammatory response is desirable, limiting the inflammatory event to the site of infection or tissue damage and ensuring its resolution in a timely manner in order to prevent disease. However, local cytokine production has been demonstrated to activate
the systemic and hepatic acute phase protein (APP) response, resulting in the production of the APPs serum amyloid A, C-reactive protein, α1-acid glycoprotein and haptoglobin among others (Gruys, Toussaint et al. 2005). These APPs possess a wide range of functions, including the ability to act as opsonins in order to initiate the complement cascade and the ability to limit the growth of microbes (Gabay and Kushner 1999). The ultimate aim of the APP response is to limit infection/tissue damage and resolve the inflammatory event in a timely manner. The persistence and spread of the inflammatory event beyond the site of infection can result in an overwhelming systemic inflammatory response which is detrimental to the host and can cause sepsicaemia (Bosmann and Ward 2013). In addition, prolonged inflammation locally, as a result of continuous antigenic exposure or a result of the failure to resolve, can result in chronic inflammation which is marked by tissue damage and ultimately progression to disease. The events leading to chronic inflammation locally are currently poorly understood.

While the process of inflammation has received connotations of being a pathological process in the past, the concept is now emerging that pathological inflammation has a physiological counterpart, providing a tightly regulated inflammatory response to prevent infection and maintain tissue homeostasis.

1.2 Physiological and pathological inflammation

The concept of physiological and pathological inflammation was initially established in relation to the intestinal mucosa (Fiocchi 2003). Within the intestine, low levels of inflammation are continuously observed in the intestinal mucosa, with accumulation of immune cells observed in organised immune structures such as the Peyer’s patches or dispersed throughout the intestinal epithelium (Mowat 2003). This is considered a ‘healthy’ or ‘physiological’ inflammatory immune response, required to maintain homeostasis and is essential to normal gut health. Physiological inflammation is characterised by its ability to adapt to pro-inflammatory challenges, discriminating between foreign and commensal bacterial antigens (or MAMPs) and mounting an appropriate immune response to deal with the threat, a response that is highly controlled and resolved in due course. Thus, the concept of physiological inflammation as a normal response that prevents tissue injury has emerged (Fiocchi 2008). When the ability to
maintain physiological inflammation is lost, the result is a switch to ‘pathological inflammation’, resulting in tissue damage and ultimately progression to disease.

The point at which physiological inflammation becomes pathological and the events mediating this switch from physiological to pathological inflammation remain poorly defined and understood. However, pathological inflammation is known to result from an excessive, inappropriate and uncontrolled response to inflammatory stimuli, resulting in tissue damage and acute or chronic disease. The difference in acute versus chronic disease is marked by the ability and time taken to resolve this pathological disease. Inability to resolve chronic pathological inflammation ultimately results in prolonged and more severe tissue damage and poorer disease outcome, as evidenced by the chronic pathological inflammatory response observed in Crohn’s disease (Rubin, Shaker et al. 2012).

The switch to pathological inflammation can largely be attributed to the host’s inability to maintain physiological inflammation, be this as a result of a persistent inflammatory stimulus, the inadequate production of resolution mediators or the failure of a phenotypic switch in either macrophage or other regulatory immune cell populations. A number of studies have proposed the switch from physiological to pathological inflammation at mucosal surfaces may be linked to the loss of epithelial barrier integrity, indicating the central role of the epithelium at mucosal surfaces (Karin, Lawrence et al. 2006).

As we gain a greater understanding of the molecular mechanisms surrounding inflammatory processes, our previous view of inflammation as a pathological reaction to infection or injury that needs to be suppressed needs updating. Additionally, while the concept of physiological and pathological inflammation has been established in relation to the intestinal mucosa, it has particular relevance at other mucosal, sites and in particular the female reproductive tract (FRT) where the process of parturition and subsequent postpartum mechanisms of tissue repair and involution represent a major inflammatory event. This concept of physiological inflammation has been largely ignored in studies involving the FRT to date.

1.3 Inflammation at mucosal surfaces
As mentioned previously, mucosal sites such as the intestine, lungs and reproductive tract interact constantly with the external environment, with almost continual exposure to immunological stimulation from commensal microorganisms and potential invading pathogens. Mucosal surfaces are crucial sites for both innate and adaptive immune regulation, maintaining the balance between tolerance of commensals and the ability to appropriately respond to pathogenic challenge.

The FRT must deal with the dual challenge of fighting off infection from invading bacteria and viruses while maintaining a tolerant environment allowing for successful reproduction (Mor, Cardenas et al. 2011). The FRT is composed of a number of anatomically distinct regions. The lower part of the FRT is composed of the vagina and ectocervix while the upper FRT is composed of the endocervix, the endometrium and ovaries. Each region has distinct functions with regard to reproduction and immune protection as well as possessing very different microbiomes.

Across species, the mucosal barrier of the uterus is of particular interest due to its dual role of fighting infection and maintaining pregnancy. Following parturition, bacteria invade the uterus resulting in a shift in the uterine microbiome causing an inflammatory immune response. The mucosal barrier of the uterus has developed multifaceted defence mechanisms to overcome these threats and return the organ to a state of homeostasis. These include the production of host-defence peptides (HDPs), pro-inflammatory cytokines and chemokines. The cellular components of the FRT play a crucial role in mediating these defence mechanisms and maintaining a physiological level of inflammation.

### 1.4 Anatomy of the uterine mucosal barrier

The bovine uterus is described as having a bipartite shape consisting of two horns (Frandson, Wilke et al. 2009). The mucosal tissue lining the uterus is termed the endometrium, the thickness and vascularity of which vary depending on the stage of the oestrous cycle and on pregnancy. Forming a selectively permeable barrier between the host and the external environment are epithelial cells which are responsible for the production of mucins which form a mucus gel that overlies the epithelium and forms a physical barrier preventing large molecules and bacteria from coming in direct contact.
with the epithelium. Underlying the epithelium is the remaining functional layer of the endometrium which is mainly composed of stromal fibroblasts. The stromal fibroblasts being in closer contact with the blood supply are capable of releasing and responding to pro-inflammatory and sex-steroid signals before the epithelium (Figure 1.1). Protruding from the endometrium in mushroom-like projections are the caruncles which form the maternal part of the foetal-maternal interface during pregnancy, fusing with the foetal cotyledons to form placentomes (Frandsen, Wilke et al. 2009).

Figure 1.1: Anatomy of the bovine female reproductive tract.

The bovine uterus has a bipartite shape consisting of two uterine horns, the inner layer of which is termed the endometrium. A cross section of the endometrium shows epithelial cells forming a selectively permeable barrier overlying the stroma which is in close contact with the blood supply. Overlying the epithelium is a layer of mucus which functions to separate the epithelium form the commensal microbiome and invading pathogenic microbes.
The structure of the endometrium is under the control of the oestrous cycle, which is defined as the periods of ovarian activity in which female animals transition from a period of reproductive non-receptivity to a period of receptivity with the ultimate aim of establishing pregnancy (Forde, Beltman et al. 2011). Cattle are poly-oestrous, having a cycle every 21 days. The cycle consists of two phases, the luteal phase which is dominated by progesterone and lasts between 14-18 days during which the corpus luteum is formed and the follicular phase which is dominated by estradiol, lasts between 4-6 days and follows the demise of the corpus luteum until ovulation occurs where the oocyte is released into the oviduct for fertilisation. The cycle is under the control of hormones released by the hypothalamus, the pituitary gland and the ovaries (Crowe 2008, Forde, Beltman et al. 2011).

While primarily being responsible for control of the oestrous cycle, sex steroid hormones have also been implicated in regulating the innate immune responses of the FRT. Progesterone concentrations, found at their highest during the luteal phase, have been shown to play an immunosuppressive role allowing for the development of uterine infections (Rowson, Lamming et al. 1953, Lewis 2003). Del Vecchio et al (1994) found that cows that received intrauterine infusions of bacteria when progesterone concentrations where basal did not develop uterine infections, while cows who received infusions of bacteria when progesterone concentrations had begun to increase all developed uterine infections (Del Vecchio, Matsas et al. 1994). To counteract the immunosuppressive effects of progesterone, the bovine FRT has developed a number of innate immune defences to allow it achieve its reproductive function while also maintaining an immune function. These effector functions are largely mediated by the innate cells that comprise the endometrial tissue and provide its structure.

1.4.1 Mucus

The endometrium’s first line of defence is the secreted mucus. Composed of mucin glycoproteins, the mucus functions to trap and transport debris and invading bacterial pathogens, preventing their attachment and invasion into the tissue (Johansson and Hansson 2016). Within the gut the mucus acts as a diffusion barrier, allowing it to trap high levels of IgA, particularly in the duodenum and ileum (Macpherson, McCoy et al.
Within the human FRT, mucin gene expression varies according to the stage of the menstrual cycle, leading to changes in the composition and overall properties of the mucus (Gipson, Ho et al. 1997). Mucus in the human FRT has also been shown to protect epithelial cells from direct contact with viral pathogens such as HIV (Shukair, Allen et al. 2013). Mucus has also been reported as being a rich source of immunological molecules, including cytokines and APPs (Adnane, Meade et al. 2018).

The importance of mucus has been highlighted in a number of chronic diseases including cystic fibrosis and ulcerative colitis, although reports differ when categorizing mucus as playing a role in either healthy or pathological inflammation. One study looking at mucus production in a mouse model of ulcerative colitis and in human patients found that defects in Muc2 production was associated with an increased ability of bacteria to penetrate the inner mucus layer of the colon allowing access to the epithelium (Johansson, Gustafsson et al. 2014). Conversely, mucin hyper-production has been associated with many airway associated diseases such as asthma and cystic fibrosis where its overproduction can impair mucociliary clearance of pathogens and also plug airways (Kang, Hwang et al. 2015). Within the FRT mucus has been found to plays a reproductive role, with loss of Muc1 expression required to create a receptive surface for embryo attachment (Bowen, Bazer et al. 1996).

### 1.4.2 Epithelial cells

Epithelial cells line the endometrium and form a selectively permeable barrier between the external environment and the underlying stroma. In contrast to the lower FRT which is lined by stratified squamous epithelium, the upper FRT (which includes the endometrium) is lined by a single layer columnar epithelium (Wira, Rodriguez-Garcia et al. 2015). Epithelial cells have been defined based on the expression of certain cytoskeletal proteins including cytokeratin, E-cadherin and β-catenin among others (Franke, Schiller et al. 1981, Kalluri and Weinberg 2009); while other studies have found their distinct cuboidal/columnar morphology allows them to be easily identified when viewed under the microscope (Fortier, Guilbault et al. 1988). An increasing body of evidence is emerging demonstrating that epithelial cells mediate immunological responses to microbes through the production of a wide array of cytokines, host-defence peptides and acute phase proteins.
The primary role of epithelial cells is to provide a physical barrier to the external environment. They are highly adapted and specialized in order to accomplish this task which is mediated through the apical junctional complex, composed of the tight junction and the sub-adjacent adherens junction and desmosome (Table 1.1) which together maintain tight links between epithelial cells (Turner 2009).

Table 1.1: Components of the apical junctional complex.

The apical junctional complex, responsible for maintaining the selectively permeable barrier between epithelial cells can be divided into three distinct regions: the tight junction, the adherens junction and the desmosome. These regions can be further subdivided into the interacting proteins that form the junctions (Turner 2009).

<table>
<thead>
<tr>
<th>Region of apical junctional complex</th>
<th>Components</th>
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<tbody>
<tr>
<td>Tight Junction</td>
<td>Claudin</td>
</tr>
<tr>
<td></td>
<td>Occludin</td>
</tr>
<tr>
<td></td>
<td>ZO-1</td>
</tr>
<tr>
<td></td>
<td>F-Actin</td>
</tr>
<tr>
<td></td>
<td>Myosin</td>
</tr>
<tr>
<td></td>
<td>MLCK</td>
</tr>
<tr>
<td>Adherens Junction</td>
<td>E-Cadherin</td>
</tr>
<tr>
<td></td>
<td>α-Catenin</td>
</tr>
<tr>
<td></td>
<td>β-Catenin</td>
</tr>
<tr>
<td>Desmosome</td>
<td>Desmoglein</td>
</tr>
<tr>
<td></td>
<td>Desmoplakin</td>
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<tr>
<td></td>
<td>Desmocollin</td>
</tr>
</tbody>
</table>

The apical junctional complex forms the bonds that maintain cell-cell contact and is essential for regulating the paracellular pathway which allows the passive movement of molecules through the space between adjacent cells (as opposed to the transcellular pathway which involves the active movement of molecules across the cell membrane) (Figure 1.2). The apical junctional complex also gives rise to the trans-epithelial electrical resistance (TER) property of epithelial cells which can be used to measure the barrier function of epithelial cells grown on porous supports in culture (Powell 1981). This property of TER can be harnessed when growing epithelial cells in-vitro to investigate the establishment of the apical junctional complex and the resulting process of cell polarisation. To assess the TER, an electrical current is passed through the transwell support containing the epithelial cells, resulting in a continuous current passing through both the transcellular and paracellular pathway. The resistance measured across
the epithelium is composed of the resistance provided by the apical and basolateral cell membranes and by the paracellular resistance provided by the cell-cell contact of the apical junctional complex (Chen, Einspanier et al. 2015). This measurement of paracellular resistance allows for the identification of physical structures such as the apical junctional complex and can be utilised to investigate the establishment of tight junction complexes and the permeability of the epithelial barrier (Narai, Arai et al. 1997).

The apical junctional complex maintains barrier function and its dysregulation has been associated with several inflammatory intestinal diseases and is strongly linked to the ‘switch’ to pathological inflammation. The importance of the tight junction in regulating mucosal immune responses has been demonstrated in a mouse model of colitis (Su, Shen et al. 2009). Disruption of the epithelial barrier results in the activation of mucosal immune responses resulting in the onset of immune mediated colitis in this model. Loss of barrier function has also been implicated in the pathogenesis of human Crohn’s disease (Zeissig, Burgel et al. 2007). Patients with Crohn’s disease have impaired intestinal barrier functions, indicated by a distinct reduction in epithelial resistance. The downregulation of claudins 5 and 8 also resulted in an altered tight junction structure and further impaired the barrier function of the intestine in these patients.
While epithelial cells in the thymus have the ability to present processed antigen to naïve T cells, their immunological role in peripheral organs such as the uterus have been relatively ignored in favour of their physical barrier function. Within the intestine, epithelial cells secrete host defence peptides and mediate the transcytosis of secretory IgA into the intestinal lumen, while also producing immune-regulatory signals to instruct mucosal immune cells of the intestine (Peterson and Artis 2014). Specialised epithelial cells known as goblet cells have been shown to produce mucus. While the immune role of epithelial cells in the uterus has been less well defined, our group and others have shown epithelial cells lining the bovine FRT express a wide array of pathogen recognition receptors (PRRs) allowing them to respond to microbial threats through the production of cytokines, chemokines and HDPs (Aflatoonian, Tuckerman et al. 2007, Davies, Meade et al. 2008).

The ability of the epithelial cells to form the apical junctional complex allows the epithelial cells to undergo a process of cell polarization, defined as the asymmetrical distribution of proteins, lipids and cell components. This allows for epithelial cells to...
recognise and differentially respond to antigens and cell signals based on whether they are presented to the cells’ apical or basolateral membrane and localize different activities to distinct regions of the cell (Figure 1.2). This is of particular importance when examining immune responses as the direction in which immune molecules are secreted from the epithelium will have a major impact on their role in an inflammatory response.

Epithelial cells have been shown to be able to produce a wide spectrum of pro-inflammatory cytokines and chemokines. A review by Swamy et al (2010) highlighted the importance of molecules used by epithelial cells to instruct other immune cells and coined the term epimmunome, emphasizing the important role epithelial cells play in the immune response (Swamy, Jamora et al. 2010). Within the bovine endometrium investigations have been limited mainly to IL-6 and IL-8 production, with polarized endometrial epithelial cells predominantly secreting IL-6 apically in response to bacterial stimulation, with this secretion mediated via the trans-golgi network. In contrast IL-8 was not secreted directionally but was secreted from both the apical and basolateral membranes (Healy, Cronin et al. 2015).

In addition to the pro-inflammatory cytokines and chemokines, epithelial cells are among the main producers of host defence peptides (HDPs). While the mechanisms surrounding inflammatory cytokine production by epithelial cells are poorly defined, HDP expression has been relatively well defined. These peptides have potent anti-microbial and immunomodulatory properties contributing to the innate immune responses of the reproductive tract (Matsuzaki 1999). These roles can often appear contradictory and their role as either anti- or pro-inflammatory molecules remains to be decided. HDPs are short cationic peptides that form amphipathic conformations that allow them to interact with membranes (Ganz 2003). HDPs can be subdivided into a number of families based on their structure, including defensins, cathelicidins, whey-acid proteins and S100 proteins.

Defensins are the largest family of HDPs and can be sub-divided into α-, β- and θ-defensins based on the location and organization of their six cysteine bonds (Lehrer and Lu 2012). α-defensins are primarily produced by neutrophils in human and are not present in the bovine. β-defensins comprise the most widely studied subgroup of defensins and are the only defensin subgroup found in bovine while θ-defensins exist only in primates. There are four human β-defensins which are primarily produced by epithelial cells (Zhao, Wang et al. 1996, Tunzi, Harper et al. 2000) and have been found to be highly expressed
at sites in the human FRT. Our group has previously identified an expansion of the β-
defensin repertoire in the bovine (Cormican, Meade et al. 2008). Work in the human has
shown that β-defensins possess strong immunomodulatory properties with β-defensin-2
and -3 being chemotactic for CD4⁺ T cells, neutrophils and macrophages among others
(Rohrl, Yang et al. 2010).

Cathelicidins are so called because of the highly conserved N-terminal coding region
called the cathelin domain which was named after its ability to inhibit the cysteine
protease cathepsin L (Zanetti, Gennaro et al. 1995). The bovine genome contains multiple
cathelicidin genes and previous work by our group has quantified expression of these
cathelicidins in a panel of bovine tissues and in an experimental model of bovine mastitis
(Whelehan, Barry-Reidy et al. 2014). Cathelicidin-5 (CATHL5) is the sole bovine
cathelicidin expressed in the uterus and the most highly expressed bovine cathelicidin
gene in the FRT. Constitutive expression of CATHL5 mRNA across several regions of
the mammary gland in healthy cows and its induction in Staphylococcus aureus infected
cows suggest a role for CATHL5 in protection against mastitis.

Whey acid proteins (otherwise known as the WAP-four disulphide core (WFDC)
proteins) have also been shown to have a role in the FRT. From this family of HDPs,
secretory leukocyte protease inhibitor (SLPI) and elafin have been shown to be expressed
in the human FRT. SLPI has been shown to have roles in promoting wound healing
(Ashcroft, Lei et al. 2000), while SLPI deficient mice were found to have expansive
wounds caused by unresolved inflammation and a failure of re-epithelization.

It is evident that epithelial cells possess a potent immunological repertoire and that their
potential contribution to inflammatory cytokine production has gone largely un-
investigated. The epithelial barrier is routinely lost or disrupted during the process of
parturition resulting in the exposure of the underlying stroma immediately postpartum,
resulting in the need for stromal fibroblasts to possess an equally effective immunological
repertoire.

1.4.3 Stromal fibroblasts

Underlying the epithelium and lying in close contact to the endometrial blood supply,
endometrial stromal fibroblasts (also termed stromal cells) have classically been
dismissed as having purely structural functions. Stromal cells possess a spindle-shaped morphology which is formed by their expression of cytoskeletal proteins such as vimentin, N-cadherin and P-cadherin (Kalluri and Zeisberg 2006). There is some conflict regarding the definition of a stromal cell, with some reports defining a stromal cell as any cell type below the epithelium including endothelial cells and pericytes. Here, we define stromal cells as stromal fibroblasts. Stromal fibroblasts have been attributed a wide range of functions, summarised in Figure 1.3.

![Figure 1.3: Overview of stromal fibroblast functions in the endometrium](image)

Stromal fibroblasts have been found to have multifactorial roles within the endometrium. While some aspects such as the ability to provide structure to organs have long been appreciated, other aspects such as the ability to regenerate are only now being elucidated. Their ability to regulate epithelial function, drive inflammation and respond to stimulus, be it pathogenic or steroid, have confirmed their role as key mediators of the endometrial immune response.

Being in closer proximity to the uterine blood supply allows endometrial stromal fibroblasts to be among the first to receive and respond to signals transmitted through the blood. Stromal fibroblasts rather than epithelial cells respond to sex steroid signalling as many of the sex steroid receptors (including those for progesterone and estradiol) are expressed primarily in the stroma as opposed to the epithelium (Bigsby and Cunha 1986). Our lab has previously identified that stromal fibroblasts from patients with
endometriosis produced significantly less stem cell factor (required for NK cell development) compared to stromal fibroblasts from healthy controls following stimulation with progesterone or oestrogen resulting in an impaired fertility phenotype (Thiruchelvam, Wingfield et al. 2016). Work by our group and others has also demonstrated that bovine endometrial stromal fibroblasts express TLRs 1-4, 6, 7, 9 and 10; indicating a role for stromal cells in responding to PAMPs encountered in the FRT (Davies, Meade et al. 2008).

Stromal fibroblasts are among the key producers of the extracellular matrix (ECM) which provides the support and act as scaffolds for tissues to carry out their functions (Palumbo Jr, Ferreira et al. 2012). Stromal fibroblasts play roles in tissue tropism, localizing inflammation to specific sites. They achieve this through the production of cytokines and chemokines and through the expression of cell adhesion molecules such as VCAM-1 (Feuerbach and Feyen 1997). Stromal fibroblasts located in the mesenteric lymph node have also been shown to be essential for the generation of gut-homing T cells through the induction of gut-homing molecules such as α4β7-integrin (Hammerschmidt, Ahrendt et al. 2008).

Stromal fibroblasts play a role in maintaining homeostasis and differentiation of the adjacent epithelium. This has been exemplified in the mammary gland where stromal-epithelia interaction is vital for the organization and development of the mammary gland (Wiseman and Werb 2002). The mammary gland develops into its adult form by a process known as branching morphogenesis. Stromal fibroblasts are required to stimulate this initial branching to occur (Simian, Hirai et al. 2001). Disruption of this stromal-epithelial crosstalk in the mammary gland has been found to promote the development of breast cancer (Xu, Langenheim et al. 2012).

Faced with tissue destruction during parturition and the requirement for tissue remodelling and reduction in size of the uterus postpartum, the uterus is a highly regenerative organ. Owing to stromal fibroblasts being of mesenchymal origin, they possess stem cell or progenitor capabilities, allowing them to play a role in uterine regeneration (Donofrio, Franceschi et al. 2008, de Moraes, Maia et al. 2016). Bovine endometrial stromal fibroblasts are indistinguishable from bone marrow derived stromal fibroblasts based on morphology and on the analysis of cytokines secreted. Bovine endometrial stromal fibroblasts could be differentiated along an osteogenic lineage when
cultured in an osteoblastic differentiation media. This was confirmed by a change in morphology and positive staining for calcium deposits (Donofrio, Franceschi et al. 2008). A population of ovine endometrial stromal fibroblasts have also been shown to differentiate into four mesodermal lineages including adipocytes, osteoblasts, chondrocytes and smooth muscle cells (Letouzey, Tan et al. 2015).

The immunological properties of stromal fibroblasts are evidenced by the role they play in the accumulation of pro-inflammatory lymphoid cells. During a normal inflammatory immune response stromal fibroblasts produce cytokines and chemokines and express cell adhesion molecules to attract immune cells to the site of inflammation. When the inflammation is resolved the stromal fibroblasts return to their resting phenotype, devoid of cytokine production. However during periods of prolonged pathological inflammation, stromal fibroblasts acquire changes in their phenotype due to prolonged cytokine secretion and mediate the formation of tertiary lymphoid organs (Buckley, Barone et al. 2015). Tertiary lymphoid organs (TLOs) form in peripheral tissues during chronic inflammatory immune reactions where infiltrating leukocytes become organized into well-defined lymphoid like structures. TLOs function to orchestrate the local immune response and are associated with chronic pathological inflammatory conditions (Butler, Cosgrove et al. 2017).

As mentioned, stromal fibroblasts contribute to inflammatory responses through the production of cytokines and chemokines and through the presentation of antigen. Investigations into cytokine production by stromal fibroblasts has been limited to a select number of inflammatory cytokines and so, similar to endometrial epithelial cells, detailed mechanisms of activation and regulatory mechanisms surrounding cytokine production are lacking. Stromal fibroblasts are potent producers of IL-6 in response to DAMPs such as hyaluronan, HMGB1 and IL-1α (Healy, Cronin et al. 2014). Stromal fibroblasts also produce IL-8 and IL-1α in response to LPS stimulation. The production of these cytokines and chemokines in response to LPS or *E. coli* stimulation was found to be independent of ovarian steroids or stage of the oestrous cycle from which they were isolated (Saut, Healey et al. 2014). Stromal fibroblasts have been shown to be the target for many viruses targeting the FRT such as bovine herpesvirus-4 and thus have evolved to develop a strong anti-viral immune response (Jacca, Franceschi et al. 2013). Emerging work from our group has also demonstrated β-defensin expression in response to viral stimuli by stromal fibroblasts (Barry-Reidy et al-in preparation).
While it is clear that stromal fibroblasts have been largely attributed a purely structural role, evidence is now emerging of an immunological role for this cell population. A thorough characterisation of their response to inflammatory stimuli and the role they play in inflammation is now warranted.

1.4.4 Myeloid and lymphoid cell populations in the endometrium

Characterisation of the immune cell repertoire in the bovine endometrium has been greatly restricted due to the lack of commercially available reagents such as antibodies for bovine research. However, it is clear from work carried out on the human FRT that the endometrium possesses a unique immune cell repertoire capable of fighting infection, maintaining pregnancy and tissue repair and remodelling.

CD3+ T cells comprise only 1-2% of the immune cell population in the human endometrium (Salamonsen and Woolley 1999). In contrast to what is found in the peripheral blood, endometrial CD3+ T cells consist of a larger proportion of CD8+ T cells (66%) with a smaller proportion of CD4+ T cells (33%). These T cell populations were found to be distributed in the basal lymphoid sites and scattered throughout the stroma and epithelium. B cells are also present within the human endometrium, but in low numbers (Salamonsen and Lathbury 2000). The presence of T and B cells has been confirmed in the bovine endometrium by immunofluorescence (Oliveira, Mansourri-Attia et al. 2013).

Previous work by our group has characterised uterine Natural Killer (uNK) cell function and phenotype within the human FRT (Lysakova-Devine and O'Farrelly 2014). uNK cells demonstrate altered expression of surface markers, are weakly cytotoxic and are potent cytokine producers (McGrath, Ryan et al. 2009). We have also shown that uNK cells play a significant role in successful human pregnancy, with abnormal uNK cell maturation contributing to infertility in women with endometriosis (Thiruchelvam, Wingfield et al. 2016). uNK cells have also been shown to have a role in tissue remodelling in the mouse and human (Robson, Harris et al. 2012).

Macrophages are present in the human endometrium across all stages of the menstrual cycle and are primarily located around the glands. Tissue resident macrophages have classically been associated with roles including phagocytosis, wound healing,
maintenance of tissue homeostasis and regulation of other immune cell populations (Wynn and Barron 2010). Macrophages within the endometrium have been shown to be an important source of both pro- and anti-inflammatory cytokines (Thiruchelvam, Dransfield et al. 2013). During the proliferative phase of the human menstrual cycle, endometrial macrophages have been shown to express adhesion and activation markers such as CD71, CD69 and CD54 implicating them in a potential role in regeneration of the functional layer of the endometrium (Eidukaite and Tamosiunas 2004). During the secretory phase of the menstrual cycle macrophage numbers increase. Production of macrophage products such as MIP-1β, MIF and CSF-1 that are fundamental in creating the inflammatory phenotype present at this stage of the cycle are also increased (Kats, Al-Akoum et al. 2005, Thiruchelvam, Dransfield et al. 2013).

In the human, neutrophils have been found in endometrial tissue easily identified by their morphology and comprise 6-15% of the total cell number at days 27-28 of the menstrual cycle. The importance of the neutrophil in the endometrium is evidenced in a mouse model where endometrial breakdown and repair is artificially induced. Depletion of neutrophils using the antibody RB6-8C5 resulted in a marked delay in endometrial repair. Neutrophils were the most abundant cell type found during tissue repair, with macrophages only barely detectable during tissue repair and uNK cells only found in intact decidua (Kaitu'u-Lino, Morison et al. 2007).

Within the bovine, neutrophils are recruited into the endometrium in the immediate postpartum period in response to the chemokine IL-8. The level of infiltrating neutrophils is used to diagnose subclinical endometritis. While cut-off values for PMN infiltration seem to vary in the literature, most sources agree on a cut-off of approximately >18% PMN infiltration for the diagnosis of SCE from cytobrush samples (Kasimanickam, Duffield et al. 2004). As mentioned previously, neutrophils migrate into the endometrium in response to the chemokine IL-8 which is segregated into two distinct haplotypes in the bovine. Previous work by our group has found that IL-8 haplotype can result in variation in the systemic immune response to LPS in Holstein-Friesian calves and is likely to account for some of the observed inter-breed and inter-animal response to infection (Stojkovic, McLoughlin et al. 2016).
It is clear that there is a significant gap in our knowledge of immune cell populations within the bovine endometrium, compromising our understanding of the endometrial immune response.

1.5 Barrier function and inflammation postpartum

The events of parturition and the subsequent process of tissue remodelling results in a significant level of disruption to the mucosal barrier of the endometrium. Physical damage during parturition often results in the sloughing off of the epithelial layer, exposing the stroma. The process of uterine involution in the postpartum period can also result in significant disruption to the mucosal barrier with the requirement for physical shrinkage and the resulting necrosis and sloughing of the caruncles (Sheldon, Williams et al. 2008). This disruption to the endometrial barrier also coincides with a disruption in the uterine microbiome. Taken together, the disruption to the mucosal barrier and dysregulation in the uterine microbiome leave the endometrium vulnerable to infection and inflammation.

Dysregulation of the uterine microbiome in the postpartum period has been described in detail in the literature and with advances in culture-independent methods of microbial characterisation, our understanding of the contribution of the microbiome to uterine disease can only improve. Additionally, the gravid uterus, previously thought to be sterile, has recently been shown to possess its own distinct microbiome (Karstrup, Klitgaard et al. 2017). As regards uterine disease, previous reports have identified distinct differences existing in the bacterial populations present in healthy cows and cows that had cytological endometritis (Knudsen, Karstrup et al. 2016) A study by Jeon et al (2015) found that the microbiota changed rapidly in the first week postpartum with an increase in the abundance of Bacteroides and Fusobacteria in cows that went on to develop metritis. The increase in abundance of these bacteria also correlated with an increase in uterine discharge score (Jeon, Vieira-Neto et al. 2015). Work by Wagener et al (2014) found that the postpartum uterine microflora was dominated by the presence of Trueperella pyogenes (13.2%) and Escherichia coli (11.2%) and the presence of T. pyogenes increased the vaginal discharge scores used to diagnose uterine disease. E. coli was the predominant species present within the first week postpartum while T. pyogenes dominated from the second week postpartum onwards (Wagener, Prunner et al. 2014).
We are now beginning to appreciate a reduction in the diversity of microbial populations in cows with uterine disease and it is clear that this dysregulation of the uterine microbiome has implications for the establishment of uterine disease. Here we discuss the immune response of the endometrium to this dynamic inflammatory environment.

Studies examining endometrial immune function normally employ a reductionist approach, investigating the responses of individual cell populations to stimulations with PAMPs or DAMPs. However, endometrial architecture is completely disrupted in the isolation of these individual cell populations and the fact that these cell populations do not function in isolation within the endometrium. Thus, examining the response of mixed cell populations or intact endometrial explants may provide a more relevant picture of what is occurring *in-vivo* in the postpartum period. The immune response of endometrial explant tissue and mixed cell populations is summarised in *Table 1.2*. 
<table>
<thead>
<tr>
<th>Study</th>
<th>Cytokines examined</th>
<th>Result</th>
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<tbody>
<tr>
<td>Explants of intact endometrium to model bovine innate immunity and inflammation ex vivo. (Borges, Healey et al. 2012)</td>
<td>IL-6, IL-8 &amp; IL-1β.</td>
<td>Endometrial explants accumulated IL-6, IL-8 and low levels of IL-1β (2-6 pg/mg tissue) in response to gram-positive and gram-negative bacteria and their purified PAMPs. Cytokine responses were found to be dependent on the stage of the oestrous cycle.</td>
</tr>
<tr>
<td>Time course of defence mechanisms in bovine endometrium in response to lipopolysaccharide. (Swangchan-Uthai, Lavender et al. 2012)</td>
<td>IL-1β, TNF, IL-8.</td>
<td>A time course of LPS stimulation of mixed endometrial epithelial and stromal cells found TNF peaked first at 1hr with IL-1β peaking at 6hrs. IL-8 displayed one of the highest fold changes in mRNA expression in response to LPS stimulation.</td>
</tr>
<tr>
<td>Endometrial cells sense and react to tissue damage during infection of the bovine endometrium via interleukin 1. (Healy, Cronin et al. 2014)</td>
<td>IL-6, IL-8, IL-1β.</td>
<td>Endometrial explants accumulated significantly higher levels of IL-6 in response to stimulation with IL-1α. Explants also accumulated low levels of IL-8 while IL-1β was not detectable.</td>
</tr>
<tr>
<td>Ovarian steroids do not affect bovine endometrial cytokine or chemokine responses to Escherichia coli or LPS in-vitro. (Saut, Healey et al. 2014)</td>
<td>IL-6, IL-8, IL-1β.</td>
<td>Co-stimulation of endometrial explants with LPS/E. coli and oestradiol or progesterone or inhibitors of oestradiol or progesterone nuclear receptors did not affect IL-6, IL-8 or IL-1β production.</td>
</tr>
<tr>
<td>Global transcriptomic profiling of bovine endometrial immune response in vitro. Effect of lipopolysaccharide on innate immunity. (Oguejiofor, Cheng et al. 2015)</td>
<td>Whole transcriptome.</td>
<td>Mixed endometrial cell populations were stimulated for 6hrs and responses measured by microarray. 30% of genes altered by LPS were involved in the immune response. Cytokines and chemokines such as IL-1 and CCL5 were among the most upregulated.</td>
</tr>
<tr>
<td>Glutamine modulates inflammatory responses to lipopolysaccharide in ex vivo bovine endometrium. (Noleto, Saut et al. 2017)</td>
<td>IL-6, IL-8, IL-1β.</td>
<td>Reducing the availability of glutamine reduced the production of IL-1β, IL-6 and IL-8 by approximately 50%. Inhibiting glycolysis reduced accumulation of cytokines by approximately 50%, even when glutamine and glucose are abundant.</td>
</tr>
</tbody>
</table>
While studies involving explant tissue can provide an important link to what is occurring in the animal *in-vivo*, the response of single cell populations can provide an important insight into the role both epithelial and stromal fibroblasts play in mediating endometrial inflammation. Within the field of bovine research, the isolation and culture of endometrial epithelial and stromal cells has been pioneered by Prof. Martin Sheldon’s research group in Swansea University. His work examining the immune responses of endometrial epithelial and stromal fibroblasts is summarised in Table 1.3. His research has focused on the mechanisms surrounding the production of IL-6 and IL-8 by both cell populations.
Table 1.3: Summary of immune responses observed in pure populations of bovine endometrial epithelial and stromal cells.

<table>
<thead>
<tr>
<th>Study</th>
<th>Epithelial and Stromal Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coordinated Role of Toll-Like Receptor-3 and Retinoic Acid-Inducible Gene-I in the Innate Response of Bovine Endometrial Cells to Virus. (Carneiro, Bedford et al. 2017)</td>
<td><strong>Epithelial:</strong> Treatment with BVDV induced IL-6 and IL-8 expression. Treatment with BoHV-4 reduced IL-6 production but increased expression of IL-8.  <strong>Stromal:</strong> Treatment with BVDV induced IL-6 expression but not IL-8. Treatment with BoHV-4 increased IL-8 expression but had no effect on IL-6 production.  Endometrial cell populations clearly differ in their immune response depending on the viral challenge.</td>
</tr>
<tr>
<td>Signal transducer and activator of transcription-3 licenses Toll-like receptor 4-dependent interleukin (IL)-6 and IL-8 production via IL-6 receptor-positive feedback in endometrial cells. (Cronin, Kanamarlapudi et al. 2016)</td>
<td><strong>Epithelial:</strong> IL-6R, but not STAT3 and SOCS3, is essential for LPS-induced IL-6 production in epithelial cells. Polarized epithelial cells modulate STAT3 regulation of stromal cell IL-8 production.  <strong>Stromal:</strong> Production of IL-6 and IL-8 was dependent on STAT3, SOCS3 and Src kinase signalling in stromal cells.</td>
</tr>
<tr>
<td>Polarized epithelial cells secrete interleukin 6 apically in the bovine endometrium. (Healy, Cronin et al. 2015)</td>
<td><strong>Epithelial:</strong> Polarized epithelial cells only secrete IL-6 apically in response to E. coli bacteria, LPS or IL-1α. IL-8 was found to be secreted into both the apical and basolateral compartment.  <strong>Stromal:</strong> A confluent epithelium was found to be essential in protecting the underlying stromal cells from lysis caused by the PLO PAMP. An un-confluent epithelial layer also resulted in stromal cell secretion of IL-6 into the basolateral compartment, further propagating the pathological inflammatory reaction.</td>
</tr>
<tr>
<td>Endometrial cells sense and react to tissue damage during infection of the bovine endometrium via interleukin 1. (Healy, Cronin et al. 2014)</td>
<td><strong>Epithelial:</strong> Higher levels of IL-8 were produced by epithelial cells than stromal cells in response to stimulation with DAMPs such as HMGB1 or IL-1α. IL-6 was produced but at lower levels than in the stromal cells. IL-1β was not detected in response to DAMP stimulation.  <strong>Stromal:</strong> Higher levels of IL-1α were produced by stromal cells than epithelial cells in response to stimulation with E. coli. Stromal cells also produced higher levels of IL-6 in response to stimulation with IL-1α than epithelial cells.</td>
</tr>
</tbody>
</table>
Epithelial and Stromal Cells

<table>
<thead>
<tr>
<th>Study</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides in vitro via Toll-like receptors TLR2, TLR1, and TLR6. (Turner, Cronin et al. 2014)</td>
<td>Epithelial and stromal cells produced IL-6 and IL-8 in response to triacylated or diacylated bacterial lipopeptides. The accumulation of IL-6 and IL-8 in response to triacylated lipopeptides was reduced by siRNA targeting TLR2 or TLR1 but not TLR6, whereas cellular responses to diacylated lipopeptide were reduced by siRNA targeting TLR2, TLR1, or TLR6. Stimulation with ovarian steroids had little impact.</td>
</tr>
</tbody>
</table>
| Polarised bovine endometrial epithelial cells vectorially secrete prostaglandins and chemotactic factors under physiological and pathological conditions. (MacKintosh, Schuberth et al. 2013) | Epithelial: Prostaglandins were secreted basolaterally by epithelial cells. IL-8 accumulated apically and was highly chemotactic for neutrophils. IL-8 secretion was also found to be directed towards the site of LPS treatment.  
Stromal: Co-culture of epithelial cells with stromal cells did not influence epithelial cell responses. |
| Toll-like receptor 4 and MYD88-dependent signalling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. (Cronin, Turner et al. 2012) | Epithelial: Treatment with LPS increased IL1B, IL6 and IL8 mRNA expression and increased IL-6 protein production. Knockdown of TLR4 or MYD88 using siRNA reduced the inflammatory response to LPS in epithelial and stromal cells. Inhibition of MAPK3/1, but not JNK or MAPK14, reduced LPS-induced IL1B, IL6, and IL8 mRNA expression in both epithelial and stromal cells.  
Stromal: Treatment with LPS increased IL1B and IL8 mRNA expression and increased IL-6 and IL-8 protein production. |
| Toll-like receptor and antimicrobial peptide expression in the bovine endometrium. (Davies, Meade et al. 2008) | Epithelial: TLRs 1-7 and 9 were expressed on epithelial cells. Epithelial cells also expressed the antimicrobial peptides LAP, TAP and MUC1 in response to LPS stimulation. Epithelial cells did not express detectable amounts of acute phase proteins such as SAA or haptoglobin.  
Stromal: TLRs 1-4, 6, 7, 9 and 10 were expressed on stromal cells. |
### Epithelial and Stromal Cells

<table>
<thead>
<tr>
<th>Study</th>
<th>Result</th>
</tr>
</thead>
</table>
| Bovine herpesvirus 4 is tropic for bovine endometrial cells and modulates endocrine function. (Donofrio, Herath et al. 2007) | **Epithelial:** BoHV4 was capable of infecting epithelial cells but was much more efficient at infecting stromal cells. In contrast to stromal cells, BoHV4 did not increase COX2 expression and prostaglandin E\(_2\) secretion in epithelial cells.  
**Stromal:** Co-culture of macrophages with stromal cells reactivated BoHV4 replication in persistently infected macrophages. |
As discussed previously, inflammation has developed a connotation as being pathological and requiring therapeutic intervention. However, we propose that the inflammatory response within the postpartum endometrium should be considered a normal, healthy physiological response, required to return the endometrium to a state of homeostasis allowing it to prepare itself for future conception (Chapwanya, Meade et al. 2012). The switch from physiological to pathological inflammation as evidenced in uterine disease is still poorly understood despite our greater appreciation for the immunological properties of epithelial cells and stromal fibroblasts. Elucidating the mechanisms driving this switch is crucial to the development of targeted immunotherapies for treating pathological inflammatory immune conditions in the FRT. In cattle, mRNA transcripts for potent pro-inflammatory cytokines such as IL-1β have been found to be more abundant in the endometrium of cows suffering from endometritis than in healthy animals, leading us to question what role this cytokine plays in the endometrial immune response (Sheldon, Cronin et al. 2014, Foley, Chapwanya et al. 2015).

1.6 IL-1β and the inflammasome

Considered among the most potent molecules of the innate immune system is the IL-1 family of cytokines. Principal among these is IL-1β, which is produced in response to infection and during tissue damage (Sims and Smith 2010). The discovery of IL-1β represented a seminal advance in the field of inflammatory cytokines and is crucial to our understanding of inflammatory pathology today (Dinarello 2010). Since the 1940s, reports had circulated of a secreted protein which resulted in the rapid onset of fever when injected into rabbits (Beeson 1948). This molecule was termed human leukocytic pyrogen (LP). In 1972, the discovery of Lymphocyte activating factor (LAF), a product secreted by macrophages, was reported (Gery and Waksman 1972). With increasing awareness that LP and LAF possessed very similar properties, it was reported in 1979 that both described the same molecule, which was later renamed as IL-1 (Rosenwasser, Dinarello et al. 1979). IL-1 has since been attributed many diverse properties, including the ability to induce hepatic acute phase protein expression and activate lymphocytes (Merriman, Pulliam et al. 1977). This was a highly controversial concept at the time, that a single molecule could possess such a wide range of functions, with lengthy debate as to whether this was a result of the activity of multiple proteins with similar molecular weights due
to the lack of an amino acid sequence or a recombinant protein for IL-1 to allow for experimental testing. The reporting of the IL-1 cDNA sequence in 1984 by both Lomedico et al and Auron et al revolutionised the study of this inflammatory cytokine, although the sequence identified by Lomedico was later revealed to be IL-1α (Auron, Webb et al. 1984, Lomedico, Gubler et al. 1984). The identification of the cDNA sequence for IL-1β resulted in the production of the recombinant IL-1β protein allowing for experiments to demonstrate and confirm the wide range of effector functions possessed by the cytokine Later work examining its regulation and requirement for processing for its biological activity still has implications for inflammation and the study of inflammatory diseases to date.

Since its discovery in the 1980s the IL-1 family has grown to include the members IL-1α, IL-1β, IL-18, IL-33, IL-1 receptor antagonist and IL-1F5-IL-1F10 (summarised in Table 1.4). IL-1β is predominantly secreted by dendritic cells, macrophages and monocytes (Eriksson, Kurrer et al. 2003, Netea, Nold-Petry et al. 2009). Evidence has also emerged of its production by neutrophils and epithelial cells (although epithelial cells are considered the main producers of IL-33) (Schmitz, Owyang et al. 2005).
Table 1.4. Summary of IL-1 family members.

Protein size, processing enzymes, associated receptor complexes and regulatory factors for the IL-1 family of cytokines are summarised. Alternative names for IL-1 cytokines are given in parentheses. Summarised from (Dinarello 2010, Sims and Smith 2010).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Pro-form Size</th>
<th>Active-form Size</th>
<th>Processing enzyme</th>
<th>Receptor Complex</th>
<th>Regulating factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>30.6 kDa</td>
<td>17 kDa</td>
<td>Calpain</td>
<td>IL-1R1</td>
<td>IL-1RA IL-1R2 SIGIRR</td>
</tr>
<tr>
<td>(IL-1F1)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1RAP</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>35 kDa</td>
<td>17 kDa</td>
<td>Caspase-1</td>
<td>IL-1R1</td>
<td>IL-1RA IL-1R2 SIGIRR</td>
</tr>
<tr>
<td>(IL-1F2)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1RAP</td>
<td></td>
</tr>
<tr>
<td>IL-1RA</td>
<td>20 kDa</td>
<td>17 kDa</td>
<td>Signal peptidase</td>
<td>IL-1R1</td>
<td>IL-1RA IL-1R2 SIGIRR</td>
</tr>
<tr>
<td>(IL-1F3)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1RAP</td>
<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>24 kDa</td>
<td>18 kDa</td>
<td>Caspase-1</td>
<td>IL-18Rα IL-18Rβ</td>
<td>IL-18BP SIGIRR</td>
</tr>
<tr>
<td>(IL-1F4)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1RAP</td>
<td></td>
</tr>
<tr>
<td>IL-36RA</td>
<td>N/A</td>
<td>17 kDa</td>
<td>N/A</td>
<td>IL-1Rrp2</td>
<td>SIGIRR</td>
</tr>
<tr>
<td>(FIL-1δ)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1RAP</td>
<td></td>
</tr>
<tr>
<td>(IL-1F5)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1Rrp2</td>
<td></td>
</tr>
<tr>
<td>IL-36α</td>
<td>N/A</td>
<td>20 kDa</td>
<td>N/A</td>
<td>IL-1Rrp2</td>
<td>IL-1F5 SIGIRR</td>
</tr>
<tr>
<td>(FIL-1ε)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1RAP</td>
<td></td>
</tr>
<tr>
<td>(IL-1F6)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1Rrp2</td>
<td></td>
</tr>
<tr>
<td>IL-37</td>
<td>24 kDa</td>
<td>19 kDa</td>
<td>Unknown</td>
<td>IL-18Rα</td>
<td>Unknown</td>
</tr>
<tr>
<td>(IL-1H4)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1RAP</td>
<td></td>
</tr>
<tr>
<td>(IL-1F7)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1Rrp2</td>
<td></td>
</tr>
<tr>
<td>IL-36β</td>
<td>N/A</td>
<td>18.5 kDa</td>
<td>N/A</td>
<td>IL-1Rrp2</td>
<td>IL-1F5 SIGIRR</td>
</tr>
<tr>
<td>(IL-1H2)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1RAP</td>
<td></td>
</tr>
<tr>
<td>(IL-1F8)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1Rrp2</td>
<td></td>
</tr>
<tr>
<td>IL-36γ</td>
<td>N/A</td>
<td>20 kDa</td>
<td>N/A</td>
<td>IL-1Rrp2</td>
<td>IL-1F5 SIGIRR</td>
</tr>
<tr>
<td>(IL-1ε)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1RAP</td>
<td></td>
</tr>
<tr>
<td>(IL-1F9)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1Rrp2</td>
<td></td>
</tr>
<tr>
<td>IL-38</td>
<td>25 kDa</td>
<td>17 kDa</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>(IL-1Hy2)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1RAP</td>
<td></td>
</tr>
<tr>
<td>(IL-1F10)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1Rrp2</td>
<td></td>
</tr>
<tr>
<td>IL-33</td>
<td>30 kDa</td>
<td>18 kDa</td>
<td>Unknown</td>
<td>ST2</td>
<td>Soluble ST2 SIGIRR</td>
</tr>
<tr>
<td>(IL-1F11)</td>
<td></td>
<td></td>
<td></td>
<td>IL-1RAP</td>
<td></td>
</tr>
</tbody>
</table>

Following its production and release by the cell, IL-1β acts on the IL-1 receptor which is composed of two subunits; IL-1R1 and its accessory protein IL-1RACP which is recruited following ligand binding to IL-1R1. Formation of this receptor heterodimer allows for the propagation of further downstream signalling through the NFκB and MAPK pathways (Korherr, Hofmeister et al. 1997). IL-1β has been shown to act on a variety of both innate and adaptive immune cells resulting in increased cytokine production in both
dendritic cells and macrophages, increased phagocytosis in macrophages and promotes increased survival and oxidative burst and protease release in neutrophils (Wright, Moots et al. 2010). IL-1β has also been implicated in driving TH17 cell differentiation resulting in the production of IL-17 (Chung, Chang et al. 2009).

Being such a potent pro-inflammatory cytokine, the release of IL-1β from cells is a tightly regulated process. Initially produced as a 35kDa precursor protein following a priming signal through the TLR4 signalling pathway, IL-1β is required to be cleaved into its active 17kDa form to allow it to be released from the cell. This cleavage is mediated through the inflammasome signalling complex (Martinon, Burns et al. 2002). Since its discovery the inflammasome family has grown to include the nod-like receptor family of inflammasomes which includes NLRP1B, NLRP3 and NLRC4, and the pyhin family of inflammasomes which includes the AIM2 and IFI16 inflammasomes. In addition to the canonical inflammasome pathways there is also a non-canonical inflammasome pathway which directly targets caspases in the absence of an inflammasome receptor. Other, less well characterized, inflammasomes include the NLRP6, NLRP7, NLRP12 and RIG-I inflammasomes.

NLRP3 is the main inflammasome complex implicated in the response to microbial challenge and its activation requires two signals. The first comes in the form of a PAMP such as LPS which is recognised through the TLR4 receptor complex and results in the transcriptional priming of pro-IL-1β and of the NLRP3 inflammasome complex. The second signal comes through the recognition of an inflammatory signal through the inflammasome receptor complex. For the NLRP3 inflammasome these signals are varied and include DAMPs such as extracellular ATP and hyaluronan; markers of metabolic stress such as extracellular glucose and monosodium urate crystals and microbial toxins such as nigericin (summarised in Table 1.5) (Schroder and Tschopp 2010). Despite this varied collection of stimuli the common step caused by these signals is potassium efflux from the cell which is sensed by the NLRP3 receptor (Petrilli, Papin et al. 2007). A number of reports have emerged identifying a role for NEK7 (a protein previously associated with mitosis) in NLRP3 activation (He, Zeng et al. 2016, Shi, Wang et al. 2016). NEK7 acts downstream of potassium efflux and has been found to be required for NLRP3 activation and oligomerisation and achieves this by binding to the leucine rich repeats of the NLRP3 receptor (He, Zeng et al. 2016).
Table 1.5. NLRP3 inflammasome activating agents.

Summary of the varied stimuli with proven roles in NLRP3 inflammasome activation (Jo, Kim et al. 2016).

<table>
<thead>
<tr>
<th>Agent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nigericin</td>
<td>Microbial toxin</td>
</tr>
<tr>
<td>Extracellular ATP</td>
<td>DAMP</td>
</tr>
<tr>
<td>Urate Crystals</td>
<td>Environmental crystal</td>
</tr>
<tr>
<td>Aluminium Hydroxide</td>
<td>Environmental crystal/adjuvant</td>
</tr>
<tr>
<td>Silica</td>
<td>Environmental crystal</td>
</tr>
<tr>
<td>Asbestos</td>
<td>Environmental crystal</td>
</tr>
<tr>
<td>Staphlococcus Aureus</td>
<td>Bacterial pathogen</td>
</tr>
<tr>
<td>Group B Streptococcus</td>
<td>Bacterial pathogen</td>
</tr>
<tr>
<td>Listeria Monocytogenes</td>
<td>Bacterial pathogen</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>Bacterial pathogen</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>Polysaccharide</td>
</tr>
<tr>
<td>Extracellular glucose</td>
<td>Sugar</td>
</tr>
</tbody>
</table>

Activation and oligomerisation of the NLRP3 receptor results in the recruitment and oligomerisation of the adaptor protein ASC. ASC is composed of a pyrin (PYD) and a caspase activation and recruitment domain (CARD). The PYD domain is used to interact with the PYD domain of the NLRP3 receptor while the CARD domain is used to interact with the CARD domain of caspase-1. In this way ASC acts as a bridge lining the NLRP3 receptor to the cleavage of caspase-1 (Mariathasan and Monack 2007). Following expression within the cell, ASC molecules oligomerise to form discrete specks, with typically one speck per cell (Proell, Gerlic et al. 2013). This complex then mediates the cleavage of caspase-1 from its pro-form to its active form which in turn acts on pro-IL-1β, mediating its cleavage into its active form allowing it to be released from the cell. Caspase-1 also mediates the cleavage of gasdermin D into its active form, which has implications for IL-1β release and pyroptotic cell death (He, Wan et al. 2015). This process is summarised in Figure 1.4.
Figure 1.4. Mechanism of NLRP3 inflammasome activation.

Following LPS priming through the TLR pathway the NLRP3 inflammasome complex will respond to stimuli such as ATP or Nigericin. This results in NLRP3 oligomerisation (promoted by NEK7) and recruitment of the ASC adaptor complex. ASC in turn mediates the cleavage of pro-caspase-1 into its active form. Active caspase-1 can then mediate the cleavage of pro-IL-1β into its active form, allowing its release from the cell. Active caspase-1 also mediates the cleavage of gasdermin D into its active form, which in turn travels to the cell membrane, forming pores and resulting in pyroptotic cell death.

As mentioned above, the final step in inflammasome activation is the cleavage of pro-IL-1β into its active form by the caspase-1 enzyme. Caspase-1 belongs to the caspase family of cysteine proteases which are initially synthesized as inactive zymogens composed of a large and small subunit which must be cleaved to become active (Thornberry, Bull et al. 1992). Effector caspases also contain either a caspase activation and recruitment (CARD) domain or a death effector domain (DED) which allows them to be recruited and interact with other proteins (Bouchier-Hayes, Conroy et al. 2001). The caspases as a family are broadly divided into two groups based on their role in either inflammation or programmed cell death. While the inflammatory caspases have been found to play a role
if the inflammasome complex they have also been shown to induce pyroptosis, a lytic form of cell death following IL-1β cleavage (Man and Kanneganti 2016). In contrast to the highly pro-inflammatory pyroptotic form of cell death, the other broad group of caspases mediate apoptosis, a form of programmed cell death that is immunologically silent (McIlwain, Berger et al. 2013). The involvement of the inflammatory caspases in the process of pyroptosis has major implications for the mechanism underlying the release of IL-1β from the cell.

IL-1β lacks a signal peptide and its release from the cell does not follow the classical ER-Golgi route of secretion. The mechanism of IL-1β release from the cell remains highly debated with a number of different theories proposed, with most reports concluding that IL-1β secretion is mediated through a spectrum of mechanisms (Lopez-Castejon and Brough 2011). There is evidence of IL-1β being targeted to lysosomes for degradation, which can subsequently be rescued by triggering lysosome exocytosis and thus secretion of IL-1β (Andrei, Dazzi et al. 1999). Another demonstrated route of expression is via the shedding of microvesicles from the plasma membrane. Reports have found that the IL-1β contained in the microvesicles is bioactive and may be released following contact with cells expressing the IL-1 receptor (MacKenzie, Wilson et al. 2001). Stimulation of these microvesicles with the inflammasome activating agent ATP also induces release of their contents (Bianco, Pravettoni et al. 2005). However, investigations have mainly focused on the role of gasdermin D and the process of pyroptosis in mediating IL-1β release (Hogquist, Nett et al. 1991). Following inflammasome activation, gasdermin D is cleaved into its active form by caspase-1 (He, Wan et al. 2015). Gasdermin D can then travel to the cell membrane where it forms pores, resulting in pyroptotic cell death and IL-1β release. A recent report has demonstrated that IL-1β maturation enables its relocation to the cytosol of the plasma membrane. Caspase-1 activity was found to control the speed of IL-1β secretion via its processing of gasdermin D (Monteleone, Stanley et al. 2018).

While research into the NLRP3 inflammasome in mice has focused on the role of caspase-1, other members of the caspase family have begun to receive attention in recent years. Caspase-11, which functions in the non-canonical inflammasome, has been found to play an important role at mucosal sites such as the gut. In a mouse model of experimental colitis, caspase-11<sup>−/−</sup> mice were found to have impaired IL-18 secretion which resulted in reduced intestinal epithelial cell proliferation and increased cell death (Oficjalska, Raverdeau et al. 2015). Inflammatory caspases such as caspase-11 have also been shown
to play a role in the cleavage of gasdermin-D resulting in an N-terminal fragment that can cause pyroptosis. (Kayagaki, Stowe et al. 2015).

To date, the majority of research into the production and release of IL-1β by the inflammasome complex has been carried out using mouse models of disease. However, in recent years, evidence has been emerging of distinct differences between inflammasome activation in mice versus humans. One such report found that human monocytes engage an alternative inflammasome pathway. This pathway still relied on classical inflammasome components such as NLRP3, ASC and caspase-1 but did not display any of the classical inflammasome characteristics such as the dependency of potassium efflux to initiate inflammasome signalling, pyroptosome formation and the induction of pyroptosis (Gaidt, Ebert et al. 2016). Other observed differences between human and mouse inflammasome activation include the observation that signal one alone is enough to induce inflammasome activation in primary human monocytes (Wang, Mao et al. 2013). Other sources have attributed this to constitutive caspase-1 expression, although whether this could be as a result of the physical or chemical stress caused during the isolation of these primary human monocytes is debatable (Netea, Nold-Petry et al. 2009). Human monocytes also appear more sensitive to LPS than mouse monocytes, with higher concentrations of LPS (>100 ng/ml) capable of acting as the second signal for inflammasome activation (Chin and Kostura 1993).

Despite the discrepancies that exist between human and mouse inflammasome pathways, the research into both mouse and human has been extensive in comparison to the bovine. Similar to human and mouse, the bovine inflammasome regulates the cleavage and release of IL-1 family members in response to classical inflammasome stimuli such as LPS and alum in bovine PBMCs and in response to LPS and ATP in bovine monocytes (Hussen, Düvel et al. 2012, Harte, Gorman et al. 2017). Despite the similarities in function and activation, some species specific differences do exist. Bovine monocytes do not require the ATP-gated ion channel P2X7 for inflammasome activation, despite inflammasome activation requiring ATP and potassium efflux as the second signal in addition to LPS priming (Hussen, Düvel et al. 2012). Caspase-8 has been implicated in mediating IL-1β cleavage in bovine PBMCs (Harte, Gorman et al. 2017). Species specific differences also exist with regard to the AIM2 inflammasome which responds to viral stimuli such as double stranded DNA resulting in the cleavage and release of IL-1 (Rathinam, Jiang et al. 2010). The AIM2 gene in the bovine is pseudogenised, rendering
it non-functional (Cridland, Curley et al. 2012). However, evidence is emerging to suggest that another pyhin family member, IFI16 may function to sense double stranded DNA adjuvants (Dr. Ciaran Harte, pers. comm.). Breed specific differences in IL-1β production have also been identified between the high milk yielding dairy cow breed Holstein Friesian and the beef breed Brown Swiss, with the Holstein-Friesian dairy breed found to produce more IL-1β in response to bacterial or fungal ligands than its Brown-Swiss counterpart (Gibson, Woodman et al. 2016).

Notable advances in the field of inflammasome research include the discovery of a small molecule inhibitor of the NLRP3 inflammasome. Aberrant activation of the NLRP3 inflammasome has been implicated in a number of conditions including cryopyrin associated periodic syndrome, multiple sclerosis and type II diabetes and the need for an effective drug to target the inflammasome has gained increased attention in recent years (Masters, Simon et al. 2009). Recently, the development of a small molecule inhibitor of the NLRP3 inflammasome called MCC950 has been reported (Coll, Robertson et al. 2015). This molecule is capable of blocking canonical and non-canonical NLRP3 activation at nanomolar concentrations, reducing IL-1β production in-vivo. The molecule was specific in its inhibition of NLRP3 and had no effect on the AIM2, NLRC4 or NLRP1 inflammasomes. In addition, MCC950 was shown to attenuate the severity of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, and also reduced neonatal lethality in a mouse model of CAPS (Coll, Robertson et al. 2015). Another interesting advance in IL-1β research to emerge is the finding that IL-1β can function as an innate immune sensor of microbial proteolysis resulting in further IL-1β production. They hypothesised that this role might predate the evolution of the inflammasome complex, which may come as no surprise given that IL-1β dates back to fish while the inflammasome complex is only found in mammals. LaRock et al further demonstrated that a cysteine protease SPeB from a group A Streptococcus has the ability to cleave IL-1β at a different site (the phenylalanine at position 105) than where caspase-1 cleaves (the aspartic acid at position 116) and still yields an active cytokine (LaRock, Todd et al. 2016). A recent paper has also described how neutrophils are capable of secreting proteases which can cleave and activate IL-1 cytokines, including IL-1α, IL-1β, IL-33, IL-36α, IL-36β and IL-36γ (Clancy, Sullivan et al. 2018). This is of particular importance in bovine endometritis which is diagnosed based on high numbers of
neutrophil influx into the endometrial tissue and uterine lumen, with potential implications for alternative processing of IL-1β in endometritis pathology.

A role for inflammasome activation and the release of IL-1 family members, and in particular IL-1β, in the maintenance of mucosal homeostasis has begun to emerge in recent years. The role of the IL-1 family member IL-18 in driving pathological inflammation at mucosal surfaces has previously been demonstrated, linking epithelial cell IL-18 production in the intestine to the breakdown of barrier integrity in a mouse model of colitis (Nowarski, Jackson et al. 2015). Deletion of IL-18 and its receptor in intestinal epithelial cells conferred protection from colitis. The pathological role of IL-18 was further reinforced with the discovery that deletion of the IL-18 negative regulator, IL-18bp, resulted in severe colitis and inhibited goblet cell maturation resulting in impaired mucus production that further drove the pathology of colitis (Nowarski, Jackson et al. 2015). The intestinal microbiota-associated metabolites taurine, histamine and spermine have demonstrated the ability to modulate the NLRP6 inflammasome signalling pathway, thereby affecting downstream IL-18 secretion and anti-microbial peptide production. Inflammasome inhibition was found to result in a distorted AMP profile allowing for the colonisation of a colitis-driving microbiome (Levy, Thaiss et al. 2015).

Within the FRT, evidence is emerging that IL-1β plays a role in regulating many functions at the foetal-maternal interface. IL-1β has been detected in human, mouse and bovine endometrium and embryos (McMaster, Newton et al. 1992, Simon, Piquette et al. 1993, Paula-Lopes, de Moraes et al. 1998). The expression of IL-1β and IL-1R1 was examined in bovine endometrium and in embryos, with IL-1β found to be strongly expressed in luminal and glandular epithelium. Their results also suggest the bovine blastocysts are capable of secreting and responding to IL-1β (Correia-Alvarez, Gomez et al. 2015). This finding is in line with other reports that found that the concentration of IL-1β in embryo culture conditioned media positively correlated with successful implantation indicating a role for IL-1β in driving a healthy, physiological inflammatory response (Baranao, Piazza et al. 1997). In contrast, IL-1β has also been shown to drive a pathological inflammatory response within the FRT. Higher concentrations of recombinant IL-1β have been found to inhibit stromal cell proliferation in the bovine endometrium (Davidson, Tiemann et al. 1995). It is clear from the literature that IL-1β has been attributed many conflicting roles within the FRT and its exact role in driving either physiological or pathological inflammation in the postpartum endometrium.
remains to be elucidated. In addition, the events surrounding the regulation of IL-1β secretion by the inflammasome complex remains poorly understood.

1.7 Endometritis and fertility

The Irish beef and dairy industry is centred on a seasonal based grazing system to allow for maximum profits. The calving season is focused on springtime calving to maximise peak grass growth which is utilised as a cheap food source to meet the high energy demands of the lactating cow. Delayed calving results in lower milk yields and increased feeding costs. To maintain this tight timeframe for calving, cows are required to have one calf every 365 days. The period of time between one calving and the next from the same cow is recorded as the calving interval and is used as a measure of fertility. Cows that have problems conceiving are culled and replaced with heifers so as to reduce costs (Dillon, Berry et al. 2006). In Ireland, the dairy industry primarily uses the high milk producing Holstein Friesian breed.

In the past, the breeding of dairy cows has focused on traits such as milk production to the detriment of fertility. This has been reflected in calving interval scores. Calving intervals in Ireland have increased (detrimentally) between the years 1980-2011 (Figure 1.5) while genetic merit for milk production experienced a rapid increase. However, since the introduction of an emphasis on fertility traits into the Economic Breeding Index in Ireland in the early 2000s, Ireland has seen a reduction in the length of calving interval since 2012 (Prof. Mark Crowe, pers. comm.). This has led some to proclaim that infertility within the Irish dairy herd is no longer a problem. Interestingly, in the period 2008-2016 the percentage of cows culled in a herd has increased year on year, from 13.6% in 2008 to 28.0% in 2016 (ICBF 2017). One of the main reasons for the culling of cows within dairy herds is infertility. While cows with poorer genetic merit have been removed from the national herd, post-partum uterine infections and the prolonged inflammatory phenotypes as seen in conditions such as endometritis are now being implicated in infertility. This is exemplified in a study which examined differences in endometrial gene expression between cows selected for high and low fertility, with the most highly differentially expressed genes all relating to the immune response (Moore, Pryce et al. 2016).
Figure 1.5: The trend in calving interval in Ireland since 1980.

Calving interval, defined as the period between one calving and the subsequent calving by the same cow, has increased year on year since 1980 until 2012 which saw a reversal of the trend. Data source: ICBF and (Berry, Wall et al. 2014).

Following calving, all cows experience a level of damage to the endometrium caused by the process of parturition and by the processes of tissue repair and involution. All cows also experience dysregulation and a reduction in the diversity of the uterine microbiome. In addition to this, postpartum cows are also in a state of negative energy balance where the nutritional demands of lactation exceed nutritional intake and this has detrimental impacts of the post-partum immune response (O'Neill, Kishton et al. 2016). The epithelial and stromal fibroblasts of the endometrium respond to these insults by producing a host of pro-inflammatory molecules including cytokines, chemokines and host-defence peptides which result in the recruitment of immune cells into the endometrium. This is a normal, healthy inflammatory immune response required to return the tissue to a state of homeostasis in preparation for future conception (Chapwanya, Meade et al. 2012).

An excessive or prolonged period of pathological inflammation ultimately results in uterine disease, which is initially defined as metritis. Approximately 40% of cows develop clinical metritis which is infection of the cavity, lining and deeper layers of the uterus and is characterized by brown watery discharge from the uterus, fever and reduced milk yield. If this inflammation persists beyond 21 DPP it is defined as endometritis (also
defined as sub-clinical endometritis or cytological endometritis), which is localized inflammation of the uterine lining. Rates of endometritis vary within the literature with reports indicating disease incidence varying from 5% to 75%. However most authors agree on an estimated incidence rate of approximately 20% (Bromfield, Santos et al. 2015). Endometritis is currently diagnosed based on polymorphonuclear cell (PMN) influx into the endometrium (>18% neutrophils) as quantified by cytology or histology based on cytobrushes or biopsies taken of the endometrium (Dubuc, Duffield et al. 2010). Clinical endometritis had previously been diagnosed based on the composition (>50% pus) and odour of mucus discharge from the uterus and had been considered the more severe form of uterine disease (Williams, Fischer et al. 2005). However, as we now appreciate that vaginal discharge may be a sign of inflammation in the lower reproductive tract, indicative of conditions such as vaginitis or cervicitis, and may occur in the absence of endometrial inflammation, the term previously describing clinical endometritis has been renamed purulent vaginal discharge (PVD) (Dubuc, Duffield et al. 2010). Risk factors for the development of uterine disease include retention of the placenta, dystocia and the calving environment (Sheldon, Williams et al. 2008).

Uterine disease has a number of implications for both fertility and production costs. Uterine disease has been shown to have a dramatic reduction in reproductive performance with associated implications including irregular ovarian cycles, a prolonged postpartum luteal phase and ultimately a failure to conceive (Sheldon, Williams et al. 2008, Ribeiro, Lima et al. 2013, Bromfield, Santos et al. 2015). As regards production costs, in addition to the costs associated with reduced milk yields and prolonged calving interval, endometritis has been found to result in a 1.7 fold increase in the culling rate of dairy cows compared to healthy cows (LeBlanc, Duffield et al. 2002). Current treatments for endometritis involves the use of broad spectrum antibiotics which, in the age of antibiotic resistance, are becoming more restricted and less effective in their use in animals (Kaufmann, Westermann et al. 2010). Thus the demand for targeted immunotherapies is increasing, requiring full understanding of the mechanisms driving the switch to pathological inflammation.
1.8 Thesis rationale: A role for IL-1β in pathological inflammation

Previous work by our group has used RNA-seq to investigate differences in gene expression between cows that displayed a healthy inflammatory phenotype and those that displayed a pathological inflammatory phenotype leading them to be classified as having cytological endometritis. This study found that healthy cows undergo a transition from an inflammatory phenotype at 7 DPP to a tissue repair phenotype at 21 DPP which is shown by the 4197 differentially expressed genes between the two time points. However, in animals diagnosed with endometritis, only 31 genes were differentially expressed between the two time points indicating that these animals remain in an inflammatory phenotype, as evidenced by the high expression of inflammatory genes. Several members of the IL-1 family of cytokines, including IL-1α, IL-1β and IL-18, displayed differential expression between the healthy and endometritic cows. qPCR validation of the RNA-seq dataset using the same biopsies showed significantly higher levels of IL1B mRNA at both 7 and 21 DPP in cows with endometritis compared to healthy cows (Figure 1.6) (Foley, Chapwanya et al. 2015).

![IL1B mRNA Levels](image)

**Figure 1.6:** IL1B mRNA levels in endometrial biopsies from healthy cows and cows with cytological endometritis at 7 and 21 DPP.

Endometrial biopsies were collected from cows at 7 and 21 DPP. Animals were classified as either healthy or having cytological endometritis based on uterine cytology scores. mRNA was extracted from the biopsies and reverse transcribed and levels of IL1B mRNA were quantified using qPCR. Cows with endometritis display an elevated level of IL1B mRNA expression in comparison to healthy cows (Foley, Chapwanya et al. 2015).
The group also demonstrated that IL-1β protein levels are significantly higher in cervical-vaginal mucus collected from cows with endometritis at day 7 postpartum and this elevated level of IL-1β is sustained at 21 DPP in endometritic cows compared to healthy cows (Figure 1.7) (Adnane, Chapwanya et al. 2017).

![Figure 1.7: Levels of IL-1β in cervical-vaginal mucus collected from healthy animals and animals with endometritis at 7 and 21 DPP.](image)

Cervico-vaginal mucus was collected from cows at 7 and 21 DPP. Animals were classified as either healthy or having endometritis based on uterine cytology scores. The mucus was processed and levels of IL-1β were quantified using an ELISA. Cows with endometritis display an elevated and sustained level of IL-1β in comparison to healthy cows (Adnane, Chapwanya et al. 2017).

While these reports demonstrate a high level of IL-1β expression and a strong correlation with uterine disease, sample sizes in these studies are small and no investigations were performed on individual cell populations. Thus, the cells mediating the local expression of IL-1β, the inflammasome pathways regulating its expression and its role in mediating the switch to pathological inflammation in the post-partum endometrium remain unknown. The overall objective of this thesis is to explore the potential role of IL-1β as a critical mediator of the switch to pathological inflammation and examine the possible role for the inflammasome in regulating this switch. Elucidating the mechanism of this switch is crucial to our understanding of pathological inflammation in the endometrium, uterine disease and the development of novel immunotherapies to treat bovine endometritis and associated sub-fertility.
1.9 Hypothesis and aims

Hypothesis:

*Inflammasome activation and the subsequent release of the pro-inflammatory cytokine IL-1β in endometrial cells is driving the pathological inflammatory immune response associated with postpartum endometritis in dairy cows.*

Aims:

1. Establish an appropriate culture model of bovine endometrial cells to allow for hypothesis testing.
2. Examine IL-1β production in the bovine endometrium and determine its association with endometritis pathology.
3. Determine if this IL-1β production in the endometrium is dependent on the canonical inflammasome signalling pathway for its activation and release from the cell.
Chapter 2 Materials and methods

2.1 List of reagents, materials and instruments used

Table 2.1: General reagents used with manufacturer details

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IL-8 monoclonal antibody  
Polyclonal goat anti-rabbit horseradish peroxidase  
Rabbit anti-sheep IL-8 polyclonal antibody  
Recombinant Bovine IL-8  
TMB  
**Immunostaining of endometrial cell cytoskeletal proteins**  
4’,6-diamidino-2-phenylindole (DAPI)  
**Histological processing and immunohistochemical staining of endometrial samples**  
Casein  
EnVision IHC detection kit  
Eosin  
Haemotoxylin  
Histoclear  
Hydrogen peroxide  
Magnesium sulfate  
Sodium bicarbonate  
Surgipath mounting medium  
**Flow cytometry**  
7AAD viability stain  
FAM-FLICA caspase assay kit  

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<td>Cruinn Diagnostics (Grenier)</td>
<td>Dublin, Ireland</td>
</tr>
<tr>
<td>40µm filters</td>
<td>Pluristrainer</td>
<td>Dublin, Ireland</td>
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<td>5 ml plastic pipettes</td>
<td>Cruinn Diagnostics (Grenier)</td>
<td>Dublin, Ireland</td>
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<tr>
<td>6-well flat bottom tissue culture plates</td>
<td>Cruinn Diagnostics (Cellstar)</td>
<td>Dublin, Ireland</td>
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<tr>
<td>70µm filters</td>
<td>Falcon</td>
<td>NY, USA</td>
</tr>
<tr>
<td>8mm biopsy punch</td>
<td>Kai Medical</td>
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<tr>
<td>96-well flat bottom tissue culture plates</td>
<td>Cruinn Diagnostics (Cellstar)</td>
<td>Dublin, Ireland</td>
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<tr>
<td>Cryovials</td>
<td>VWR (Nunc Inc.)</td>
<td>Radnor, PA, USA</td>
</tr>
<tr>
<td>Dissection Scissors</td>
<td>Thermo Fisher Scientific</td>
<td>Paisley, UK</td>
</tr>
</tbody>
</table>

Table 2.2: General materials used with manufacturer details
Forceps with curved teeth | Thermo Fisher Scientific | Paisley, UK
Hanging culture transwell inserts 0.4µm | Millipore | Burlington, MA, USA
Petri dishes | Greiner Bio-One | Kremsmünster, Austria
T175 Tissue culture flask | Cruinn Diagnostics (Cellstar) | Dublin, Ireland
T75 Tissue culture flask | Cruinn Diagnostics (Cellstar) | Dublin, Ireland
Vacutainers containing heparin anticoagulant | Greiner Bio-One | Kremsmünster, Austria

**Molecular biology**
96 well plates | Thermo Fisher Scientific | Paisley, UK
Optical adhesive film | Applied Biosystems (Thermo Fisher Scientific) | Paisley, UK
Stainless steel beads 5mm | Qiagen | Crawley, UK
P10/P20/P200/P1000 sterile, nuclease-free filtered pipette tips. 1.5/2.0 ml nuclease-free, sterile microcentrifuge tubes | Thermo Fisher Scientific | Paisley, UK
0.2 ml nuclease-free microcentrifuge tubes | Sarstedt | Numbrecht, Germany

**Western blotting**
Blotting paper | GE Healthcare | Buckinghamshire, UK
PVDF membrane | Biorad | Hertfordshire, UK

**ELISA**
96 well ELISA plates | Greiner Bio-One | Kremsmünster, Austria

**Immunostaining of endometrial cell cytoskeletal proteins**
EZ SLIDE 8 well glass culture slides | Millipore | Burlington, MA, USA

**Histological processing and immunohistochemical of endometrial samples**
Glass slides | Thermo Fisher Scientific | Paisley, UK
Microtome blade | Feather | Japan
Microwaveable pressure cooker | Nordic Ware | St. Louis park, MN, USA
Surgipath cassettes | Leica Biosystems | Wetzlar, Germany

**Flow cytometry**
Flow tubes | Thermo Fisher Scientific | Paisley, UK

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**Table 2.3: List of reagents used for in-vitro stimulation of cells with manufacturer details**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Company</th>
<th>Location</th>
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</thead>
<tbody>
<tr>
<td>LPS from <em>Escherichia Coli</em></td>
<td>ENZO Life Sciences (Fisher)</td>
<td>Farmingdale, NY, USA</td>
</tr>
<tr>
<td>Nigericin</td>
<td>Invivogen</td>
<td>San Diego, CA, USA</td>
</tr>
<tr>
<td>DMSO</td>
<td>Sigma-Aldrich</td>
<td>Poole, UK</td>
</tr>
<tr>
<td>IL-1β, bovine, recombinant</td>
<td>Thermo Fisher Scientific</td>
<td>Paisley, UK</td>
</tr>
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</table>
Table 2.4: List of inhibitors used with manufacturer details

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target</th>
<th>Company</th>
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<tr>
<td>Bovine caspase-4 siRNA</td>
<td>Caspase-4</td>
<td>Dharmacon</td>
</tr>
<tr>
<td>MCC950</td>
<td>NLRP3</td>
<td>Amgen</td>
</tr>
<tr>
<td>Non-targeting siRNA</td>
<td></td>
<td>Dharmacon</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>N-linked glycosylation</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Z-LEVD-FMK</td>
<td>Caspase-4</td>
<td>ENZO Life Sciences (Fisher)</td>
</tr>
<tr>
<td>Z-VAD-FMK</td>
<td>Pan-caspase</td>
<td>Invivogen</td>
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</table>

Table 2.5: List of antibodies used

<table>
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<th>Antibody</th>
<th>Supplier</th>
<th>Isotype</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Anti- bovine IL-1β</td>
<td>Biorad</td>
<td>Rabbit</td>
<td>1:750 WB; 1:75 IHC</td>
</tr>
<tr>
<td>Anti- bovine IL-1β</td>
<td>Kingfisher Biotech</td>
<td>Rabbit</td>
<td>1:750 WB; 1:75 IHC</td>
</tr>
<tr>
<td>Anti- bovine CD45</td>
<td>Kingfisher Biotech</td>
<td>Mouse</td>
<td>1: 200 IHC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Abcam</td>
<td>Mouse</td>
<td>1:1000</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Sigma-Aldrich</td>
<td>Mouse</td>
<td>1:100 IS; 1:500 WB</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>Dako</td>
<td>Mouse</td>
<td>1:100 IS; 1:1500 WB</td>
</tr>
<tr>
<td>Anti- Rabbit HRP</td>
<td>Abcam</td>
<td>Goat</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti- Mouse FITC</td>
<td>Abcam</td>
<td>Goat</td>
<td>1:250</td>
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<tr>
<td>Anti- Mouse HRP</td>
<td>Sigma-Aldrich</td>
<td>Rabbit</td>
<td>1:1000</td>
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Table 2.6: Equipment and software used with manufacturer details

<table>
<thead>
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<th>Reagent</th>
<th>Company</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuri C6 flow cytometer</td>
<td>BD Biosciences</td>
<td>Allschwil, Switzerland</td>
</tr>
<tr>
<td>Aperio ScanScope Imager</td>
<td>Leica Biosystems</td>
<td>Newcastle, UK</td>
</tr>
<tr>
<td>Aperio ScanScope Imager software</td>
<td>Leica Biosystems</td>
<td>Newcastle, UK</td>
</tr>
<tr>
<td>ChemiDoc MP System</td>
<td>Biorad</td>
<td>Hertfordshire, UK</td>
</tr>
<tr>
<td>ELISA plate reader</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial Volt-ohm meter</td>
<td>World Precision Instruments</td>
<td>Hertfordshire, UK</td>
</tr>
<tr>
<td>FlowJo software</td>
<td>FlowJo LLC</td>
<td>Oregon, USA</td>
</tr>
<tr>
<td>Gel Logic 200 imaging system</td>
<td>Kodak</td>
<td>Rochester, NY, USA</td>
</tr>
<tr>
<td>Graphpad Prism 7.00 for Windows</td>
<td>Graphpad Software Inc.</td>
<td>San Diego, CA, USA</td>
</tr>
<tr>
<td>Humidity chamber</td>
<td>Simport</td>
<td>Beloeil, Canada</td>
</tr>
<tr>
<td>Microtome</td>
<td>Leica Biosystems</td>
<td>Newcastle, UK</td>
</tr>
<tr>
<td>Nanodrop Spectrophotometer</td>
<td>Thermo Fisher Scientific</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Olympus BX51 upright microscope</td>
<td>Leica Biosystems</td>
<td>Newcastle, UK</td>
</tr>
<tr>
<td>Olympus IX81 inverted microscope</td>
<td>Leica Biosystems</td>
<td>Newcastle, UK</td>
</tr>
<tr>
<td>StepOne Plus Real time PCR system</td>
<td>Thermo Fisher Scientific</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>StepOne Software v2.3</td>
<td>Thermo Fisher Scientific</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Tecne Prime Thermocycler</td>
<td>Bibby Scientific</td>
<td>Staffordshire, UK</td>
</tr>
<tr>
<td>Tissue Lyser II</td>
<td>Qiagen</td>
<td>Crawley, UK</td>
</tr>
<tr>
<td>xCelligence real time cell analyser</td>
<td>ACEA Biosciences Inc.</td>
<td>San Diego, CA, USA</td>
</tr>
</tbody>
</table>
2.2 Bioinformatic tools

A range of publicly available bioinformatics tools were utilised to analyse the conservation of genes in the bovine and to predict protein effector function. The GenBank database (part of the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genbank) and Ensembl (https://www.ensembl.org/index.html) were used for obtaining gene and protein sequences. UCSC genome browser (https://genome.ucsc.edu/) was used to further probe genes across species. BLAST (basic local alignment tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find homologous sequences to the bovine sequences of interest. Alignment of sequences of interest was performed using ClustalOmega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic analysis was performed using MEGA-X (https://www.megasoftware.net/).

2.3 Sample collection

2.3.1 Ethics statement

All procedures described were conducted under ethical approval and experimental license from the Irish Health Products Regulatory Authority in accordance with the Cruelty to Animals Act 1876 and in agreement with the European Union (Protection of Animals Used for Scientific Purposes) regulations 2012 (S.I. No. 543 of 2012).

2.3.2 Collection and classification of endometrial biopsy samples

Endometrial biopsies had been previously collected and classified as part of a previous study (Foley, Chapwanya et al. 2015). Briefly, fifteen Holstein-Friesian cows, of mixed parity, within the same university dairy herd were sampled 7 and 21 days postpartum (DPP) in the morning after milking. A clinical examination and sample collection were conducted by a veterinarian. At each time-point, an endometrial biopsy was taken from the same post-gravid horn as previously described (Chapwanya, Meade et al. 2010). Immediately after collection, the biopsy was fixed in 10 % neutral-buffered formalin.
solution (Sigma Aldrich Poole, UK) for immunohistochemical assessment. Formalin fixed tissues were subsequently paraffin embedded.

2.3.3 Collection and classification of endometrial samples by uterine cytobrush

Samples were collected from 112 mixed parity Holstein Frisian cattle at a commercial dairy farm. Sampling was carried out in the morning after milking at 7 and 21 days postpartum. Cytobrush collection was carried as previously described (Kasimanickam, Duffield et al. 2004). Briefly, this involved trans-cervical insertion of a double guarded cytobrush into the uterus. Once the cervix was reached, the inner brush was pushed through the outer guard and rotated clockwise along the wall of the uterus three times to harvest the cells. The cytobrush was then reinserted into the inner guard and removed, and stored in a labelled cryotube on dry ice. All tubes stored on dry ice were transferred to the -80°C freezer on return to the laboratory. A second inner cytobrush was inserted whilst the outer guard was still in place which enabled the collection of cells from the same position within the uterus. The second cytobrush was rolled anticlockwise on a glass microscope slide, and later stained with Diff-Quik (a modified Wright Giemsa stain) and used to classify the cows cytologically. Veterinary records over the course of the sampling were obtained from the farmer. Reproductive records (insemination dates) were obtained from the Irish Cattle Breeding Federation (ICBF) and were used to determine the calving to conception interval and number of inseminations per cow.

Once the cytology slides had been stained and dried, they were classified by observing the number of PMNs and epithelial cells present. The slides were graded blind without knowledge of sample ID or background. A total of 200 cells were counted at 400x magnification. The animals were classified in line with recent publications, using the cut off of 18% PMN at 21 DPP (Kasimanickam, Duffield et al. 2004, Baranski, Podhalicz-Dziegielewska et al. 2012). Those with ≤ 18% PMN at 21 DPP were classified as healthy, whilst cows with > 18% PMN at 21 DPP were diagnosed with endometritis.

A vaginal mucus score was recorded for each cow at 7 and 21 DPP. Assessment of vaginal mucus was carried out using a gloved hand. A scoring system of 0-3 was used, as previously described (Williams et al., 2005). Briefly, a score of 0 or 1 was given to vaginal discharge that was clear or contained flecks of pus and the cow was classified as
healthy. A score of 2 or 3 was given to samples with over 50% purulent or muco-purulent material present in the vaginal exudate and the cow was diagnosed with purulent vaginal discharge.

2.4 Organ and cell culture

2.4.1 Cell lines

Cell culture and handling of samples was carried out according to strict standard operating procedures (SOPs). Cell culture was carried out in sterile conditions using laminar air flow hoods and sterile or autoclaved materials and reagents. The bovine endometrial epithelial (BEND) cell line (purchased from the ATCC) were cultured in vented 75cm² flasks in 5% CO₂ in air, in a 37°C incubator. Growth media consisted of 1:1 mixture of Hams F12 medium and Opti-MEM Glutamax, with 34mg/ml D-Valine, 10% heat-inactivated fetal calf serum (FBS) and 10% heat-inactivated horse serum (HS). BEND cells were sub-cultivated every 4 days with media changing every 48 hours.

2.4.2 Primary cell culture

Bovine female reproductive tracts were collected at a local abattoir within 15 minutes of slaughter. Collected tracts were identified as being free from disease and visible infection. Only tracts in the early luteal stage of oestrous (as identified by the presence of a stage 1 corpus luteum on one ovary) were used (Figure 2.1). Details of age and breed were also recorded at the time of collection.
Figure 2.1 Examples of ovary staging for tract collection.

Tracts in the early luteal stage of oestrous were identified by the presence of a stage 1 corpus luteum on one ovary.

The external surface of the tract was washed in 70% industrial methylated spirits (IMS) and the uterine horn ipsilateral to the corpus luteum was opened longitudinally with sterile scissors. The exposed endometrium was washed in PBS supplemented with 50 IU/ml penicillin, 50 IU/ml streptomycin and 2.5 mg/ml amphotericin B. The endometrial surface was dissected using a sterile scissors and forceps. Dissection was performed at the abattoir with the tissue harvested into a 50 ml tube containing 20 ml of RPMI supplemented with antibiotics as above. Samples were transported to the laboratory at room temperature within 90 mins of collection.

On arrival at the laboratory, tissue was washed twice in room temperature HBSS supplemented with antibiotics. The tissue was then chopped into fine pieces (approximately 1-3 mm³ in size) using a sharp scissors and scalpels. The tissue was then incubated in HBSS with antibiotics at 37°C for 10 mins in order to slowly return the tissue temperature to 37°C. HBSS was removed before the addition of 20 ml digestive solution (pre-warmed to 37°C). Digestive solution (100ml) consisted of 375 BAEE units of trypsin-EDTA, 50 mg collagenase II, 100 mg bovine serum albumin (BSA) and 10 mg DNase I made up to 100 ml with HBSS and 0.2 μm sterile filtered. Samples incubated with digestive solution were placed in a 37°C shaking incubator at 150 rpm for 1 hour.

The resulting mixture was filtered through a 70 μm nylon mesh cell strainer to remove any cell debris with the filtrate being collected in a tube containing HBSS with 10% FBS.
This solution was then passed through a 40 μm cell strainer to isolate the stromal cells. As the epithelial cells were too large to pass through the 40 μm cell strainer they remained on the strainer. The epithelial cells could then be isolated by turning the 40 μm cell strainer upside down and washing the epithelial cells into a clean 50 ml tube using 10 ml of HBSS supplemented with 10% FBS. Cells were then pelleted by centrifugation at 700 x g for 10 minutes. Red blood cells were lysed by re-suspending the pellet in 1 ml sterile water before addition of 4ml of complete growth media. This consisted of RPMI 1640 supplemented with antibiotics as above, 10% FBS and 1X insulin, transferring, selenium supplement with ethanolamine (ITS-X).

Cells were pelleted as before and re-suspended in complete growth media. Cells were counted using a haemocytometer with trypan blue (Sigma-Aldrich, Poole, UK) staining to distinguish live from dead cells. Cell counts were adjusted to 3×10^5 cells/ml and 8×10^5 cells/ml for epithelial and stromal fibroblasts respectively and 1ml was seeded into 75cm² vented tissue culture flasks with complete growth medium, which was stored in a 37°C incubator in 5% CO₂. Cells were allowed to reach 85% confluency before plating, with media changing every 48 hours. Visual examination of cell morphology under the light microscope was used to check purity of cultures in addition to characterisation based on expression of differing cytoskeletal proteins.

2.4.3 Explant culture

Bovine uterine explants were collected as previously described (Borges, Healey et al. 2012). The external surfaces of the uteri were washed in 70% IMS and the uterine horn opened longitudinally with sterile scissors. The exposed endometrium was washed in PBS supplemented with 50 IU/ml penicillin, 50 IU/ml streptomycin and 2.5 mg/ml amphotericin B. Tissue was collected from the inter-caruncular areas using a sterile 8 mm diameter biopsy punch and the approximately 2 mm thick translucent endometrium was then dissected from the underlying tissue using sterile scissors. Each explant was immediately transferred to HBSS supplemented with 50 IU/ml penicillin, 50 IU/ml streptomycin and 2.5 mg/ml amphotericin before being transported back to the lab.

Under sterile conditions, explants were washed a further three times in the antibiotic supplemented HBSS and then transferred to 6-well tissue culture plates so that each well
contained a single explant in 2 ml of RPMI medium supplemented with 10% FBS, 50 IU/ml penicillin, 50 IU/ml streptomycin and 2.5 mg/ml amphotericin B. The explants were orientated with the epithelial surface uppermost and cultured at 37°C with 5% CO₂. The culture medium was changed every 24 hours. Following treatments harvested supernatants were frozen at -20°C and tissue samples were weighed and stored in 2 ml of RNAlater at -80°C.

2.4.4 Polarized epithelial culture

Polarized epithelial cell cultures were prepared by seeding $0.5 \times 10^5$ cells on each hanging cell culture insert, which had previously been coated with 50 μl of matrigel diluted 1:8 in RPMI and left at room temperature for 1 hour before the remaining matrigel was aspirated. Inserts were placed in 24-well plates, with 300 μl and 800 μl of culture medium in the apical and basolateral compartment respectively. Confluence of the epithelial layer was determined by a FITC-dextran permeability assay, were 0% permeability compared to the no cell control was indicative of cells reaching confluency and polarising and by examining the trans-epithelial resistance (TER) of the epithelial barrier using the EVOM2 epithelial voltohmmeter, where high resistance (>1,000 Ωcm²) was indicative of confluence and successful polarisation. The polarized epithelial cells on cell culture inserts were treated after approximately 14 days of culture when the cells had successfully polarized.

The FITC-dextran permeability assay was performed by adding 200 μg of 4 kDa FITC-dextran to the apical compartment of the transwell insert. The plate was then incubated at 37°C with 5% CO₂ for 3 hours. Following the incubation 200 μl of media from the basolateral compartment was analysed for FITC fluorescence and compared to the no cell control for analysis. TER was measured by placing one electrode into the transwell insert while the second electrode was placed in the tissue culture well. The electrode was allowed to calibrate in the media before the resistance measurement was taken. Resistance from the blank was subtracted to give a corrected value. Values were also normalised to the area of the insert.
2.4.5 PBMC isolation and culture

Bovine peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples collected in 9 ml vacutainers containing Heparin anticoagulant. Whole blood was diluted 1:1 with HBSS and mixed gently before 25 ml was layered onto 15 ml of sterile Ficoll-paque density gradient medium in a 50 ml tube. The samples were then centrifuged at 800 × g for 25 mins with the centrifuge break turned off. The mononuclear cells were then carefully removed with a pastette into a new 50 ml tube and made up to 10 ml with wash buffer (HBSS and 5% FBS). Cells were pelleted by centrifugation at 300 × g for 5 mins. Any red blood cells carried over during the separation process were lysed by re-suspending the cells in red blood cell lysis buffer. Following the wash step and red blood cell lysis the cell pellet was re-suspended in complete RPMI media (containing 10% FBS and 1% Pen-Strep) and cells were counted using a haemocytometer and trypan blue staining. Cell were then seeded at appropriate densities in tissue culture plates containing media. The cells were incubated at 37°C with 5% CO₂ and treated within hours of plating to avoid unwanted cell death.

2.4.6 Stimulations and treatments

For time-point stimulations of epithelial cells and stromal fibroblasts, cells were plated at a density of 1.5×10⁵ cells/ml in a 24 well plate and left to rest for 24 hours before stimulation. Following the 24 hour rest period, media was removed and replaced with control media or media containing 2 µg/ml LPS for 24, 12, 6 or 3 hours before addition of 10 µM nigericin for 1 hour.

For inhibitor experiments, cells were plated at a density of 1.5×10⁵ cells/ml in a 24 well plate and left to rest for 24 hours before treatment. Cells were then treated with increasing concentrations of MCC950 (1-100 nM), Z-VAD-FMK (1-100 nM) or Z-LEV-FMK (0.02-2 µM) for 1 hour before the addition of LPS for 6 hours followed by addition of nigericin for 1 hour.

Following stimulation cells were either scraped, pelleted and lysed in RIPA buffer supplemented with protease inhibitors for protein analysis or lysed in TRIzol for mRNA
extraction and stored at -80°C. Supernatants were harvested and stored at -20°C for further analysis.

2.4.7 siRNA treatments

siRNA duplexes targeting bovine caspase-4 were designed using Dharmacon’s siDESIGN Centre. Specificity of the designed siRNA complex was confirmed by running the sequence through BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). As a control, a non-targeting siRNA (scramble) was used. Cells were seeded in a 24 well plate (stromal fibroblasts) or on hanging inserts (epithelial cells) cells to be 60-80% confluent at the time of transfection. Lipofectamine (RNAiMax) was diluted by adding 3 µl lipofectamine to 50 µl Opti-mem medium. siRNA targeting caspase-4 or non-targeting siRNA (20 uM stock) was diluted 1:50 in Opti-mem medium. The diluted siRNA was added to the diluted lipofectamine (1:1 ratio) and incubated for 5 minutes at room temperature. The siRNA complex was then added dropwise to cells (100 ul of siRNA complex added to 1ml complete RPMI media) to give a final concentration of 5 pmol siRNA in each well. Cells were incubated for 48 hrs at 37°C before the media was replaced and the transfected cells were stimulated with LPS (2 µg/ml) for 6 hours followed by addition of nigericin (10 µM) for 1 hour. Following stimulation supernatants were harvested for IL-1β analysis by ELISA and cells were harvested in TRIzol for caspase-4 mRNA expression analysis.

2.4.8 Calcium switch assay

A calcium switch assay was used to examine the effect of IL-1β treatment on the re-establishment of the epithelial barrier. High resistance (>1,000 Ωcm²) polarized epithelial cells grown on transwell inserts were used. TER was measured over time using a voltohmeter. The TER was recorded 15 mins and 5 mins prior to starting the calcium switch assay to ensure stable readings. Extracellular Ca²⁺ was chelated with HBSS containing 2mM EDTA, to disrupt the epithelial tight junctions, for 5 mins at 37 ºC, then washed three times in the apical and basolateral compartment with HBSS containing normal levels of Ca²⁺ (1.8 mM). Cells were then incubated with HBSS containing normal levels of Ca²⁺ alone or in combination with increasing concentrations of IL-1β (5-50
ng/ml). TER values were subsequently recorded every 15 mins up to 3 hours and results were plotted as a percentage of baseline TER.

2.4.9 Cell viability assays

Epithelial cell viability was examined by measuring TER, which is indicative of epithelial barrier integrity. TER was measured using an EVOM2 epithelial voltohmmeter where high resistance (>1,000 Ωcm²) indicated an intact epithelium and by default, viable cells. Stromal fibroblast viability was quantified using a Cell Titer Blue Viability Assay (Promega, Wisconsin, USA) according to manufacturer’s instructions. Briefly, cells were seeded at cells/ml in a 96 well plate. Treatments were performed and following this, media was removed and replaced with 100 µl fresh media per well to which 10 µl of Cell Titer blue dye was added. Plates were returned to the incubator for 3 hours before fluorescence was recorded at 560/590 nm.

2.5 Quantitative PCR

2.5.1 RNA extraction

Total RNA was extracted using a phenol-chloroform extraction protocol. Frozen TRIzol cell suspensions were allowed to defrost and remain at room temperature for 5 minutes. 200 µl of chloroform was added and the tubes were shaken for 15 seconds and left at room temperature for 3 mins before centrifugation at 12,000 x g for 15 mins at 4°C. At this point the samples had separated into 3 phases: an upper aqueous phase containing RNA, an interphase containing DNA and an organic phase containing proteins and lipids. 400 µl of the upper aqueous phase was transferred into a new tube containing 500 µl of isopropanol. Samples were vortexed for 30 seconds and incubated at -20°C for 30 mins. Following incubation samples were centrifuged at 12,000 x g for 10 mins at 4°C. The supernatant was discarded and the pellet containing the RNA was washed in 500 µl of 75% ethanol. The samples were then centrifuged at 8,000 x g for 5 mins at 4°C. The supernatant was removed and the pellet was air dried for 2 mins to evaporate residual ethanol before being dissolved in 40 µl nuclease free water and stored at -80°C. RNA quantity was determined by measurement of absorbance at 260 nm using a NanoDrop
spectrophotometer. A260/280 nm and A260/230 nm ratios were checked to assess presence of non-nucleic acid contamination, with ratios ≥1.8 indicating high purity. RNA quality was evaluated by 2% agarose gel electrophoresis with Sybr SAFE staining.

2.5.2 cDNA synthesis

Omniscript First Strand Synthesis kit and oligo dT primers were used for cDNA synthesis. 2 μl of 10x reaction buffer, 2 μl of 8 ng/μl of Oligo dT, 2 μl of 0.25 mM dNTP and 1 μl of Omniscript reverse transcriptase enzyme were used in a final volume reaction of 20 μl with 1 μg of RNA. The reaction was carried out in a Techne prime thermocycler, incubating at 37°C for 1 hour before a 5 min 95°C phase to denature the enzyme. cDNA solutions were diluted 1:10 in nuclease free water before storage at -20°C.

2.5.3 Primer design

Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) was used to design qPCR primers, supplying the GenBank accession number and specifying a product between 70 and 250 bp in length, which spanned an exon-exon junction to avoid amplification of any contaminating genomic DNA. All primers were synthesized by Integrated DNA Technologies in lyophilised form and re-suspended in the lab in nuclease free water and stored at -20°C. Primer details can be found in the Appendix.

2.5.4 qPCR

Quantitative real time PCR (qPCR) samples were prepared using a 10 μl reaction mix protocol: 2 μl of diluted cDNA, 5 μl of Power Up qPCR SYBR green master mix and 3 μl of a mix of primers and water. All reactions were carried out in triplicate. Primer concentration was optimised for each primer mix by titration of 100, 300, 500 and 700 nM final concentrations. All products were run on an agarose gel to confirm the presence of a single PCR product of the correct size. PCR products were sent to GATC Biotech (Konstanz, Germany) for Sanger sequencing, examining electropherograms for single peaks at each position to confirm a single amplicon and running the returned sequence
through BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure primers were specific to the gene of interest.

An Applied Biosystems Step One Plus real time quantitative PCR system was utilised to perform qPCR with the following cycle parameters: 95°C for 20 secs, 40 cycles of 95°C for 3 secs and 60°C for 30 secs, with a final melt curve stage consisting of 95°C for 15 secs, 60°C for 1 min, rising in increments of 0.3°C until a final hold at 95°C for 15 secs. A non-template control was run for each primer used in each 96-well plate to detect molecular contamination. Levels of gene of interest (GOI) expression were determined using the delta delta Ct method ($2^{-\Delta\Delta C_t}$) (Schmittgen and Livak 2008). A ratio of GOI relative to the reference gene was generated by normalising the gene of interest to a stable internal reference control gene, as determined using GeNorm analysis from a panel of possible reference genes (β-actin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A protein (PPIA), ribosomal protein S9 (RPS9) and H3 histone family member 3A (H3F3A)).

Following qPCR assays, the absence of an amplification product within the non-template control was confirmed by the absence of a plate C_q value and dissociation curves were checked for the absence of non-specific amplification as determined by the presence of a single peak.

2.5.5 GeNorm analysis

All gene amplifications were normalised to a specific gene, which was selected as the most stably expressed gene across our samples from a panel of potential reference genes (β-actin, H3F3A, GAPDH, PPIA and RPS9) using GeNorm software (version 3.4) (Vandesompele, De Preter et al. 2002). cDNA synthesised from RNA extracted from primary cultures was pooled. qPCR was performed using primers for the panel of reference genes. The Ct values obtained were used by GeNorm software to determine respective M values for each gene. M values were used to determine stable gene expression with lower M values indicating a more stable reference gene. H3F3A was found to be the most stable reference gene across the primary culture stimulations (Figure 2.2).
cDNA synthesised from RNA extracted from primary cultures was pooled and qPCR was performed using primers from a panel of reference genes. The Ct values obtained were used by GeNorm software to determine respective M values for each gene which were used to determine stable gene expression (with lower M values indicating a more stable reference gene). H3F3A was found to be the most stable reference gene from our panel.

2.5.6 Detection of leukocyte contamination of primary endometrial cell cultures via PTPRC polymerase chain reaction (PCR)

The HotStar Master Mix PCR kit was used to carry out a PCR reaction to detect transcription of the protein tyrosine phosphatase, receptor type C (PTPRC) gene, encoding the pan-leukocyte marker CD45, within endometrial cell cultures. A 10 μl reaction volume contained 0.3 μl endometrial cell cDNA, 1X CoralLoad reaction buffer, 200 μM dNTP solution, 0.3 μl HotStarTaq polymerase enzyme and 300 nM PTPRC-specific primers, with nuclease free water making up the remainder. cDNA prepared from bovine PBMCs was used as a positive control for PTPRC amplification. The constitutively expressed ribosomal protein S9 (RPS9) was amplified from all samples to ensure poor cDNA quality did not account for a lack of amplification. A non-template control without cDNA was run for both gene assays. The PCR reaction was carried out in a Techne Prime thermocycler using the following thermocycling conditions: 95°C for
5 min and 40 cycles of 95°C for 30 sec, 60°C for 1 min, 72°C for 1 min. Results were assessed by presence or absence of a DNA product of expected size on a 2% agarose gel after electrophoresis.

2.6 Western blotting

2.6.1 Cell lysis

After stimulation, cell medium was removed and pre-warmed sterile PBS was applied to the cells. The cells were scraped into a 1.5 ml eppendorf tube and centrifuged at 12,000 × g for 5 minutes to form a cell pellet. The supernatant was removed and the pellet re-suspended in 40 μl of lysis buffer. Lysis buffer was made using 1 ml RIPA buffer which contained the following protease inhibitors: leupeptin (2 μg/ml), aprotonin (2 μg/ml), sodium orthovanadate (4 μg/ml) and phenylmethylsulphonyl fluoride (100 μg/ml) and kept on ice. The lysed cells were kept on ice for 30 mins with vortexing every 10 mins. The cell pellets were then centrifuged at 12,000 × g for 5 minute at 4°C. After centrifugation, the supernatant was transferred into a fresh eppendorf tubes.

For protein extraction of cytobrush samples the brushes were lysed in 1 ml TRIzol and phase separation was performed as described for RNA extraction. For protein extraction the bottom layer was used. Any liquid overlying this layer was removed and 300 μl of 100% ethanol was added to precipitate DNA. The samples were incubated at room temperature for 3 mins before centrifugation at 2000 × g for 5 mins at 4°C to pellet the RNA. The supernatant was removed and 1.5 ml of isopropanol was added before incubation at room temperature for 10 mins to precipitate the protein. The protein was then pelleted by centrifugation at 12,000 × g for 10 mins. The supernatant was then discarded and the pellet washed in 2 ml of 0.3 M guanidine hydrochloride-95% ethanol. Samples were then incubated for 20 mins at room temperature before centrifugation at 7,500 × g for 5 mins. The supernatants were removed and the washing step was repeated twice more. Following this, 2 ml of 100% ethanol was added to the pellet, samples were vortexed and incubated at room temperature for 20 mins. Samples were centrifuged at 7,500 × g for 5 mins and the ethanol supernatant was removed and discarded. The protein pellet was left to air dry for 5 mins before being re-suspended in 100 μl 4X Laemelli
buffer with protease inhibitors (leupeptin (2 μg/ml), aprotonin (2 μg/ml), sodium orthovanadate (4 μg/ml) and phenylmethylsulphonyl fluoride (100 μg/ml)) and incubated at 50°C for 1 hour. Samples were then centrifuged at 10,000 × g for 10 mins to sediment any insoluble material and the supernatant was transferred to a fresh Eppendorf before being stored at -20°C.

2.6.2 Protein assay

A protein assay was performed using BCA Protein Assay Reagents (Pierce Biotech). BCA reagent B was diluted 1:50 in BCA reagent A and 200 μl of this solution was used per sample in a 96 well plate. 3 μl of each sample was added into the reagent mix. The plate was placed in an incubator for 30 mins at 37°C. The concentration of each sample was measured using a spectrophotometer. A standard curve was generated each time with BSA standards and the protein concentration of each sample was calculated from this. From these values, protein samples were normalised relative to the sample of lowest concentration in 20 μl dilutions. Loading buffer (5 μl) was added to each sample and boiled at 95°C for 5 mins.

2.6.3 SDS PAGE

Prior to SDS-PAGE a 15% running gel, topped with a 4% stacking gel, were prepared (see Appendix). The gels were loaded into the running tank and 1X running buffer was added and poured into the upper and lower reservoirs of the apparatus. The samples and 3μl molecular marker was used per gel. The gel was run at 110 V for 45 mins. After the run, the stacking gel was removed and the running gel retained for transfer to PVDF membranes.

2.6.4 Protein transfer

Prior to protein transfer, transfer buffer, PVDF membrane (0.2 μm pore size) and blotting pads were prepared. The transfer membrane was cut to the correct size and activated by immersion in methanol for 30 seconds and then in dH2O for 2 mins. The membrane and
blotting pads were then soaked in transfer membrane for 5 mins. Once the gel had ran, it was removed from the gel apparatus and placed in transfer buffer for 5 mins. The gel was then transferred to the transfer machine where it was sandwiched between blotting pads and a transfer membrane. The transfer machine was connected to a voltmeter and run at 10 V (250 mA) for 20 mins.

2.6.5 Blocking and probing the membrane

Once the membrane was removed from the transfer machine, it was blocked to prevent non-specific binding of antibody. Blocking was performed with 5% milk in PBS tween (PBS-T) for 1 hour. After blocking, the primary antibody was added and the blot was left rocking in the cold room overnight. Before adding the secondary antibody, the membrane was washed for 5 mins three times in PBS-T. Secondary antibodies were applied and the blot was left on the rocker for 1 hour at room temperature. The blot was then washed again for 5 mins three times, before being incubated in ECL and visualised on the Biorad ChemiDoc MP System. A mouse monoclonal to β-actin was also utilised as a loading control.

2.7 ELISA

Quantification of IL-1β was performed using the Bovine IL-1β ELISA kit according to the manufacturer’s instructions (Thermo Scientific). Samples were assayed in duplicate and measured at 450 nm with wavelength correction at 550 nm. Samples were assayed in duplicate using 50 µl per well and measured at 450 nm with wavelength correction at 620 nm. Supernatants from endometrial cell stimulations were used neat or diluted as required in reagent diluent. Cytobrush samples were diluted to a concentration of 1 mg/ml. Values were determined using four-parameter logistic regression of the standard curve.

Quantification of IL-8 was performed according to a previously reported protocol (Cronin, Hodges et al. 2014). Briefly, a 96-well ELISA plate was coated with 50 µl per well of a 2.5 µg/ml monoclonal mouse anti-ovine IL-8 antibody diluted in carbonate/bicarbonate buffer and incubated overnight on a rocker at room temperature.
The following morning the plate was washed three times in PBS-T before blocking with 150 µl/well of 4% Fish Skin Gelatin (FSG) blocking buffer for 1 hour at room temperature. A seven-point standard curve was generated by a 1 in 2 serial dilution of recombinant bovine IL-8 in 4% FSG and added to duplicate wells. Duplicate wells containing 50 µl per well of 4% FSG were included as blank wells. Cell supernatants were loaded in duplicate wells using 50 µl of sample per well. Plates were then incubated rocking for 1.5 hours at room temperature. Plates were subsequently washed as described previously and 50 µl of a 0.145 µg/ml polyclonal rabbit anti-ovine IL-8 in 4% FSG was added before incubation for 2 hours, rocking, at room temperature. Plates were washed and then incubated with 50 µl per well of 0.042 µg/ml HRP polyclonal goat anti-rabbit in 4% FSG for 1 hour. The plate was then washed and the reaction visualised by adding the substrate 3,3’,5,5’-tetramethylbenzidine (TMB). Once the standard curve had developed the reaction was stopped by the addition of 50 µl/well of 0.5 M sulphuric acid. Absorbance was measured at 450 nm with correction at 550 nm. IL-8 protein concentrations were calculated using four-parameter logistic regression of the standard curve.

2.8 Cell imaging

2.8.1 Immunofluorescent staining of endometrial cell cytoskeletal proteins

Epithelial cells or stromal fibroblasts were seeded at a density of 1×10⁵ cells/ml into 24-well tissue culture plates in complete growth medium, allowing one well each for cytokeratin staining (expressed by epithelial cells), vimentin staining (expressed by stromal fibroblasts), an IgG isotype control and an unstained well, and allowed to rest for 24 hours. Growth medium was removed, the cell monolayer was rinsed three times with PBS and fixed with 500 µl 10% neutral-buffered formalin at room temperature for one hour. Formalin was removed and the cells washed three times with PBS before the primary antibody was added. Mouse anti-human cytokeratin antibody, mouse anti-human vimentin antibody or IgG1 isotype control were applied as appropriate using 400 µl per well of a 1:100 dilution in PBS. Cells were incubated at 4°C with rocking overnight. A FITC-conjugated polyclonal goat anti-mouse secondary antibody was applied to all wells in a 1:250 dilution, incubating at 37°C for 1 hour. After washing three times in PBS,
DAPI stain to detect nuclei was applied in a 1:250 dilution for 10 minutes at 37°C. An Olympus IX81 inverted microscope was used to detect immunofluorescence, with positive cytokeratin staining in the absence of vimentin staining taken to indicate a pure endometrial epithelial cell culture and the inverse being characteristic of stromal fibroblasts.

2.8.2 Immunocytochemical staining of endometrial cell cytoskeletal proteins

Epithelial or stromal fibroblast cells were seeded at a density of 1×10^5 cells/ml or 0.5×10^5 cells/ml respectively into wells of the Millicell EZ SLIDE 8 well glass culture slides, and allowed to rest for 24 hours. Cells were washed three times in PBS before being fixed in ice-cold methanol for 10 mins at room temperature. Cells were then washed a further three times in PBS before incubation in 3% H_2O_2 for 20 mins at room temperature, followed by blocking in 10% BSA for 1 hr at room temperature. Cells were then incubated overnight with mouse anti-human cytokeratin antibody, mouse anti-human vimentin antibody or IgG1 isotype control using 100 μl per well of a 1:100 dilution in PBS. Cells were then washed three times in PBS before incubation with the secondary antibody for 30 mins at room temperature. Cells were washed a further three times before incubation with the chromogen diaminobenzidin (DAB) for 10 mins. Cells were washed a further three times before being mounted with Surgipath mounting medium. Slides were examined using an Olympus BX51 upright microscope with positive cytokeratin staining in the absence of vimentin staining taken to indicate a pure endometrial epithelial cell culture and the inverse being characteristic of stromal fibroblasts.

2.8.3 Histological processing of endometrial samples

Endometrial biopsies were removed from formalin after 24 hours incubation and immersed in 70% ethanol for 24 hours before moving them into Surgipath cassettes for overnight paraffin wax processing using the Leica TP1020 tissue processor. Biopsies were then embedded in paraffin and sectioned at 5 μm, dewaxed by immersion in histoclear and rehydrated by immersion in 100%, 70% and 50% IMS for 5 mins each. Haemotoxylin was applied to the tissue sections for 2 minutes before immersion in Scott’s tap water (30 g magnesium sulphate and 2 g sodium bicarbonate in 3 L tap water)
for 5 minutes. Tissue sections were progressively dehydrated by immersion in 50% and 70% IMS for 5 mins each before immersion in eosin for 30 seconds. Sections were then subsequently immersed in 100% IMS and two further immersions in histoclear for 5 mins each before being mounted with Surgipath mounting medium. Slides were examined using an Olympus BX51 upright microscope or imaged using the Aperio ScanScope Imager.

2.8.4 Immunohistochemical staining of endometrial tissue

Paraffin embedded endometrial biopsies were sectioned at five micrometres, incubated at 70°C overnight and dewaxed by three immersions in histoclear for 10 mins each before rehydration with 100%, 70% and 50% IMS and dH₂O for 5 mins each. Heat induced antigen retrieval using a microwavable pressure cooker was performed in sodium citrate buffer (pH 6; see Appendix), sections were then incubated in PBS for 5 mins before incubation in casein (diluted 1 in 5 with PBS) for 20 mins to block any non-specific staining. Rabbit polyclonal anti-IL-1β (1:75) or anti-CD45 (1:200) was applied overnight at 4°C in a humid chamber. Negative control sections were incubated with rabbit IgG. The following day the sections were washed twice in PBS before a peroxidase block (30% H₂O₂ diluted 1:10 in PBS) was applied for 7 mins, followed by one wash in PBS and incubation with secondary antibody attached to a peroxidase-conjugated polymer for 30 mins at room temperature. Sections were washed twice more before the substrate chromagen dianaminobenzidin (DAB) was applied for a maximum of 10 mins. The reaction was then stopped with dH₂O and washed a further two times in dH₂O before the sections were counterstained with 0.2 µm filtered haematoxylin, dehydrated and mounted with mounting medium. Slides were examined using an Olympus BX51 upright microscope or imaged using the Aperio ScanScope Imager.
2.9 Flow cytometry

2.9.1 Measuring caspase-1 activity

The FLICA™ Assay Kit was used to examine caspase-1/4 expression. Cells (epithelial cells, stromal fibroblasts or PBMCs) were seeded at a density of 0.5×10⁶ and allowed to rest overnight before stimulation with LPS (2 µg/ml) for 3 hours followed by nigericin (10 µM) for 1 hour. Following stimulation PBMCs were re-suspended and endometrial cells lifted with accutase treatment for 5 mins before centrifugation at 1,200 rpm for 5 mins to pellet the cells. Supernatants were discarded and cells were re-suspended in FACs buffer and incubated for 30 mins at 37°C with the caspase probe (FAM-YVAD-FMK). Following this, cells were centrifuged and washed 3 times in FACs buffer. The live/dead stain 7AAD was added and cells were subsequently analysed by flow cytometry using an Accuri C6 flow cytometer.

2.9.2 Gating strategy and analysis

Cells were analysed on a FSC-A vs. FSC-H plot to exclude doublets followed by FSC-A vs. SSC-A to omit debris. Dead cells were excluded using the 7AAD live/dead stain before gating on FLICA⁺ cells. Data was analysed using FlowJo software (Version 7.6.5).

2.10 Statistical analysis

Due to the small sample size of samples tested we could not assume the data followed normal distribution. Therefore, for the analysis of 2 groups where the data was independent a Wilcoxon rank sum test (Mann Whitney U test) was used. For the analysis of more than 2 groups a Kruskal-Wallis test was used. Dunns multiple comparison post-hoc test was used to correct the Kruskal-Wallis test. A p-value <0.05 was considered statistically significant. Statistical analysis was performed using Graphpad Prism 7 software.
Chapter 3 Development of an improved primary endometrial cell culture system

3.1 Introduction

The endometrium, the mucosal lining of the uterus, plays a complex role maintaining a homeostatic environment to allow for successful implantation and pregnancy while tolerating the local microbiome and also protecting against invading pathogens. The bovine endometrium is composed of a single layer of columnar epithelial cells which provide a barrier overlying the stromal matrix (Frandson, Wilke et al. 2009). While the epithelial cells are in constant interaction with immunogenic material from the external environment and commensal microbiome, during parturition and the subsequent process of tissue remodelling the epithelial layer can be disrupted. This results in stromal fibroblasts becoming exposed to microbes and endogenous DAMPs, thereby activating inflammatory pathways which can result in uterine disease.

In addition to their barrier function, epithelial cells have been shown to be potent producers of a wide selection of HDPs including defensins, cathelicidins and whey acid proteins; and pro-inflammatory cytokines and chemokines such as IL-6 and IL-8 (Narciandi, Lloyd et al. 2011, Whelehan, Barry-Reidy et al. 2014). Stromal fibroblasts have also been shown to produce a host of pro-inflammatory cytokines and play a role in defence against viral infection (Donofrio, Herath et al. 2007). Possession of such a wide repertoire of protective mechanisms demonstrates the pivotal roles that both epithelial cells and stromal fibroblasts play in the early stages of the innate immune response before immune cell recruitment (MacKintosh, Schuberth et al. 2013). However, detailed investigation into the innate immune properties of these cells is hindered by difficulty in isolating and culturing these distinct cell populations.

While in-vivo models have classically been used to capture the overall complexity of immune mechanisms, the visualisation of individual signalling pathways, particularly under a controlled stimulation environment, poses a challenge. In addition, in-vivo modelling of bovine immune responses is expensive and restricted by the large inter-animal variation due to extraneous influences (nutrition, housing etc.). In order to examine the immune mechanisms operating within the endometrium, a model that allows us to easily manipulate and control experimental variables while providing quantitative
readouts is desirable. To achieve this, we investigated the reliability of a commercially available cell line and established explant and primary cell culture models of the endometrium and investigated their relevance to the challenges posed in-vivo.

Models of endometrial cells, including the use of both cell lines, explants and primary epithelial and stromal cells, have been previously developed for use in both human and bovine (Fortier, Guilbault et al. 1988, Staggs, Austin et al. 1998, Herath, Fischer et al. 2006, Borges, Healey et al. 2012). In the bovine, a cell line model of bovine endometrial epithelial (BEND) cells is commercially available from the American Type Culture Collection (ATCC). While cell lines provide a good basis for investigation, they come with limitations including the lack of inter-animal variation observed in primary cultures and the fact they lack many characteristics of their primary cell counterparts. In addition, the genetic manipulation required to immortalise a cell can result in altered phenotype, function and responsiveness (Kaur and Dufour 2012). The problem of cell line contamination with other cell lines (such as HeLa cells) has also been an issue for nearly the past 40 years (Nelson-Rees, Daniels et al. 1981). The culture of tissue explants provides a potential link between cellular studies and in-vivo models but is restricted by the limited viability of the explants in-vitro. Primary cell cultures provide a more realistic insight into immune mechanisms, retaining the physiology and genetic makeup of their cellular counterpart’s in-vivo and demonstrating inter-animal variation which is more reflective of the whole population.

The isolation and culture of primary bovine endometrial cells has been widely reported in past literature (Herath, Fischer et al. 2006), however these reports lack detail on the isolation of pure populations. Personal communication with labs attempting to establish the primary culture model has emphasised that replicating these models has proven to be difficult and has only been achieved with a limited amount of success in the literature. Efforts by Swangchan-Uthai et al (2012) to replicate the model were met with limited success as they were unable to generate pure populations of cells and instead had to resort to using mixed cell cultures in their experiments (Swangchan-Uthai, Lavender et al. 2012). Previous work in our group has established a model of primary human endometrial stromal fibroblasts (Thiruchelvam, Wingfield et al. 2016). Taking on-board the lessons learnt from establishing the human stromal fibroblast model and discussion with other labs attempting to establish similar models, we aimed to establish an efficient, high
yielding model which would deliver pure populations of the desired endometrial cell populations.

Advances in cell biology in recent years has meant that in-vitro models have gained increasing physiological relevance to what is occurring in-vivo. The development of 3D culture models has allowed for the incorporation of multiple cell types, the inclusion of extracellular matrix materials and allows for the spatial introduction of stimulatory factors. We aimed to harness these technologies to establish a model of endometrial cells which remained closely relevant to what is occurring in-vivo and would allow us to elucidate the mechanisms driving inflammation in the post-partum endometrium.

3.2 Hypothesis and specific aims

We hypothesize that the development of a well characterised model of distinct endometrial epithelial and stromal fibroblast cell populations will allow for in-depth investigations into endometrial cell mediated inflammation.

The specific aims were to:

1. Investigate the reliability of the BEND cell line as a model of endometrial epithelial cells.
2. Establish an endometrial explant model to study innate immune responses in the intact bovine endometrium.
3. Establish and characterise a primary culture model of endometrial epithelial and stromal cells.
4. Establish a model of polarized endometrial epithelial cells.
3.3 Results

3.3.1 Characterisation of the BEND cell line

As mentioned previously, cell lines provide a cost-effective, easy to use and unlimited supply of material and represent a good basis to initiate investigations. Cell lines have been widely used in cancer research and in the pharmaceutical industry to test drug cytotoxicity and metabolism. Advantages of cell lines include the fact they provide an unlimited supply of material for study and bypass ethical concerns associated with *in-vivo* models. However as mentioned before, they bring with them certain limitations including the fact that they lack many of the characteristics of their tissue of origin.

The BEND (bovine endometrial epithelial) cell line was purchased by our group from the ATCC at passage 18. Previous reports from our lab had indicated problems with the BEND cells, with issues raised over the ability to transfect the cells and their slow growth in culture. Morphological changes across passages and differences in results from stimulations performed at different passages was observed. To combat the slow growth of the cells in culture we investigated different additions to the growth media and found that the addition of insulin helped to increase the growth rate of the cells but did nothing to stop the morphological changes that were observed across passages (*Figure 3.1*).

The BEND cells initially display the typical cobblestone morphological appearance characteristic of epithelial cells, as can be seen in passages 20-23 (*Figure 3.1*). However, following passaging the cells progressively develop a more fibroblast like morphology, losing their cobblestone morphology and appearing stringy (passages 24-31, *Figure 3.1*). A number of interventions were examined in an effort to halt this change in morphology, including changes in the tissue culture media used, changes in the density cells were seeded at or allowed to grow to confluency at and changes in the size of the flasks used, to no effect.
Figure 3.1. The BEND cell line exhibits changes in cell morphology across consecutive passages.

Following passaging $1.5 \times 10^6$ cells were seeded in a T175 cm vented flask and were grown to confluency. Cells were then imaged using an Olympus IX81 inverted microscope with camera attached. Scale bar indicates 50 μm. Passage number is included in the bottom left corner of each image.
Based on the changes in morphology we hypothesized that the BEND cells were undergoing some form of epithelial to mesenchymal transition in culture. To investigate this, we examined the mRNA expression of epithelial and stromal specific cytoskeletal markers across a number of passages. Examining the mRNA expression of keratin-18, a marker of epithelial cells, using primary epithelial cell cDNA as a positive control and primary stromal cell cDNA as a negative control, we see a complete reduction in keratin-18 expression in the BEND cells across all 12 passages examined (Figure 3.2 A.). Examining the expression of vimentin, a stromal cell marker, using primary epithelial cell cDNA as a positive control and primary stromal cell cDNA as a negative control, we see a trend towards the increased expression of vimentin as the passage numbers increase as indicated by the increased fold change values (Figure 3.2 B.).
Figure 3.2. The BEND cell line displays changes in *keratin-18* and *vimentin* gene expression across consecutive passages.

BEND cells were probed for the expression of epithelial and fibroblasts specific cytoskeletal markers. 1x10⁶ BEND cells were harvested in TRIzol following each passage. Total RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and *keratin-18* (a marker of epithelial cells) and *vimentin* (a marker of stromal cells) gene Cq values were normalised to the reference gene *H3F3A*. Columns indicate mean fold change in gene expression relative to the positive control, error bars indicate standard error of the mean (n=3).

Based on this clear change in cytoskeletal marker expression we next turned our attention to enzymes involved in regulating the epithelial-mesenchymal transition, in particular the enzyme TWIST. Relative expression of *TWIST* mRNA increased across 12 passages of the BEND cells, peaking around passage 29 (Figure 3.3). This would indicate that epithelial-mesenchymal transition is occurring within the BEND cell line at later passages and so renders them unsuitable for use in our research.
Figure 3.3. *TWIST* mRNA gene expression increases across consecutive passages in the BEND cell line.

1×10⁶ BEND cells were harvested in TRIzol following each passage. Total RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and *TWIST* (an enzyme involved in the epithelial-mesenchymal transition) gene Cq values were normalised to the reference gene *H3F3A*. Columns indicate mean relative mRNA gene expression, error bars indicate standard deviation (n=3).
3.3.2 Development of a uterine explant model

The standard approach when investigating endometrial cell-specific immune responses is to employ a reductionist approach, isolating pure populations of epithelial or stromal cells and examining their response to stimuli. This approach involves significant disruption to the tissue architecture of the endometrium which is vital to support the spatial arrangement of the cells and maintain communication between the cell populations. To combat this loss of extracellular matrix components and normal cell-cell communication pathways we decided to investigate the use of intact endometrial explants as a model to provide the link between the whole animal and cellular studies. Explants provide the closest in-vivo approach to modelling the endometrium while also maintaining the functionally important cell types in the correct spatial arrangement and maintaining other important cell types such as endothelial cells and macrophages.

We developed an explant model based upon a previously published protocol by Borges et al (2012). Explants were obtained using an 8mm sterile biopsy punch so as to reduce tissue damage and avoid the release of DAMPs which could drive sterile inflammation within our cultures. Examining the gross morphology of the explants sampled, we noted they appeared consistent with the normal structure of the bovine endometrium. The mean weight of the explants was $0.2125 \pm 0.07409$ g. Explants were maintained in 6-well tissue culture plates, submerged in 2 ml media and orientated with the luminal epithelium facing upwards (Figure 3.4 A.). Using histology to examine the explants we confirmed that the normal tissue architecture was maintained in the explants (Figure 3.4 B.). The luminal epithelium appeared intact and glandular epithelial cells were visible in the majority of explant samples with no observable disruption to the tissue.
Figure 3.4. Morphology of endometrial explants.

(A). Representative image of endometrial explants in culture medium. Endometrial explants were collected using a sterile 8 mm diameter biopsy punch. Explants were approximately 2 mm thick. 
(B). Representative cross section image of endometrial explant cultured 24 hours. Following culture explants were formalin fixed and paraffin embedded before being sectioned into samples 4µm thick using a microtome. Sections were then mounted onto glass slides, de-paraffinised and stained with haemotoxylin before being imaged under an upright microscope. Scale bar indicates 200 µm.

Viability of the explants was a major concern as it plays a role in limiting experimental design. Viability was measured using an LDH assay which measures necrotic cell death which is indicative of tissue damage within the explants. The LDH assay was unable to detect differences in LDH activity between control explants and explants stimulated with LPS and nigericin, which is surprising given the toxicity of nigericin. A more sensitive assay, such as the TUNEL assay which stains dead or dying cells, is needed in order to accurately examine the viability of the explant cultures.

Examining the constitutive expression of inflammatory cytokines in the unstimulated explant cultures, we observe significantly higher baseline expression levels of each of the inflammatory cytokines examined here (Figure 3.5). Baseline expression of IL6 mRNA was higher in explants (1.9×10^-1 relative expression units to H3F3A) compared to epithelial cells (0.8×10^-1 relative expression units) and significantly higher in explants when compared to stromal fibroblasts (0.5×10^-1 relative expression units; p=0.0280) (Figure 3.5 A.). IL8 mRNA baseline expression was higher in explants (1.8×10^0) compared to epithelial cells (0.2×10^0; p=0.0088) and stromal fibroblasts (0.3×10^0) (Figure 3.5 B.). Constitutive expression of IL1A mRNA was significantly higher in explants (0.9×10^-1) compared to epithelial cells (0.1×10^-1; p<0.0001) or stromal...
fibroblasts \((0.1 \times 10^{-1}; p=0.0027)\) (Figure 3.5 C.). Similarly, baseline expression levels of IL1B mRNA were significantly higher in explants \((2.6 \times 10^{-1})\) compared to individual cell populations \((0.004 \times 10^{-1} \text{ relative expression units in epithelial cells and } 0.005 \times 10^{-1} \text{ relative expression units in stromal fibroblasts; } p<0.0001)\) (Figure 3.5 D.).

![Graphs of IL6, IL8, IL1A, and IL1B mRNA expression](image)

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**Figure 3.5.** Endometrial explants display significantly higher baseline expression levels of inflammatory cytokines.

(A). IL6, (B). IL8, (C). IL1A and (D). IL1B mRNA baseline expression relative to H3F3A across unstimulated samples. Cells were harvested in TRizol, total RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene H3F3A. Columns indicate mean expression relative to H3F3A, error bars indicate standard error of the mean \((n=7)\). Statistical analysis was performed using a Kruskall Wallis test with Dunns multiple comparison test. \(*=p \leq 0.05; **=p \leq 0.01; ****=p \leq 0.0001.\)

We next examined the induction of these cytokines in response to a time course stimulation with either LPS alone \((2 \mu g/ml)\) or LPS in combination with the inflammasome activator nigericin \((10 \mu M)\) over a period of 24 hours. Levels of cytokine
induction in the explant tissues were low, possibly due to the high baseline level we had previously observed. Levels of \textit{IL6} mRNA were significantly elevated at 3 hours post stimulation with a mean fold-change (MFC) of 18.2 (p=0.0021) which subsided to a MFC of 1.6 at 24 hours post stimulation (Figure 3.6 A.). Similarly, levels of \textit{IL8} mRNA were significantly upregulated at 3 hours post-stimulation with a MFC of 6.8 (p=0.0012) which resolved to a MFC of 1.4 by 24 hours post-stimulation (Figure 3.6 B.). Levels of \textit{IL1A} and \textit{IL1B} were not significantly elevated, with peak \textit{IL1A} mRNA induction occurring at 6 hours post stimulation with a MFC of 4.0 (Figure 3.6 C.) and peak \textit{IL1B} mRNA occurring at 3 hours post-stimulation with a MFC of 3.8 (Figure 3.6 D.).

![Graphs showing mRNA fold change over time for IL6, IL8, IL1A, and IL1B](image)

Figure 3.6. Endometrial explants display low levels of inflammatory cytokine induction in response to LPS stimulation.

(A.) \textit{IL6}, (B.) \textit{IL8}, (C.) \textit{IL1A} and (D.) \textit{IL1B} mRNA fold change in endometrial explants following stimulation with LPS alone, for IL8 and IL6 expression analysis, or a combination of LPS and nigericin, for IL1 expression analysis. Following stimulation cells were harvested in TRIzol for gene expression analysis. Total RNA was extracted and cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene \textit{H3F3A}. Columns indicate mean fold change relative to unstimulated control, error bars indicate standard error of the mean (n=7). Statistical analysis was performed using a Mann Whitney test, comparing untreated control to a timepoint. **=p≤0.01.
While providing the closest link between the whole animal and *in-vitro* studies, explants do carry with them certain restrictions including their limited viability, problems with oxygenation and difficulties in replicating the hormonal environment of the endometrium *in-vitro*. In addition, our results here indicate that explants may be of limited value in investigating the immune response to stimuli *in-vitro*. To combat this, we aimed to establish a primary culture model of endometrial epithelial and stromal cells within our lab which would allow us to examine the cell-specific responses of the endometrium.
3.3.3 Development of primary culture model

As mentioned previously, the epithelial cells and stromal fibroblasts of the bovine endometrium are highly specialized to carry out a range of functions including providing innate defences against invading pathogens in the upper FRT. In order to elucidate the cell specific responses and signalling pathways operating in these cells, we aimed to establish a primary culture model closely resembling what is occurring in vivo. To achieve this we began to adapt a number of previously published protocols, first described by Fortier et al in 1988 (Fortier, Guilbault et al. 1988, Herath, Fischer et al. 2006).

We began collecting tracts from recently slaughtered animals. Tracts were readily available as unused by-products of the slaughtering process. Methods for optimal dissection of the tissue were initially explored. Dissection was performed on the uterine horn ipsilateral to the corpus luteum with the uterine horn dissected from the bifurcation of the uterine horns to the top of the uterine horn. The oestrous cycle stage of each tract was determined by examining the ovaries and identifying the presence of a stage I corpus luteum. Tracts in the early luteal phase of oestrous were chosen for their basal levels of progesterone, which would therefore not impact on inflammatory mediator production given that progesterone has demonstrated anti-inflammatory roles (Stites and Siiteri 1983, Butts, Shukair et al. 2007). Tracts were collected from healthy cows who were on average 91.6 months old (7.6 years) and predominantly Holstein-Friesian cows (Figure 3.7).

Figure 3.7. Age and breed of animals from which endometrium was processed for tissue culture.

Data on age and breed of animals was made available in the abattoir immediately following slaughter. (A). Histogram detailing the age categories of cows from which endometrial samples were collected, (n=41). (B). Details of the breed of cows from which endometrial samples were obtained, (n=41).
Dissection of the upper functional layer of the endometrium was optimised using a curved dissection scissors and forceps. The endometrial lining was dissected in thin strips (Figure 3.8 A.). The forceps was used to hold the edge of the endometrial lining, cutting away the fibres that tie it to the lower functional layer composed mainly of stromal fibroblasts. Harvested tissue was immediately stored in transport media. We also investigated the use of a curette, an instrument with a sharp loop at the end of a long handle commonly used in human surgery, to sample the endometrium. Tissue isolation using a curette involves running the loop over the endometrium to harvest the epithelial layer. While dissection with the curette was faster and required less skill, we found we recovered higher cell yields (particularly epithelial) by dissecting with the scissors, possibly due to less tissue being recovered using the curette (Figure 3.8 B.).

Figure 3.8. Isolation of tissue from the functional layer of the endometrium.

Isolation of endometrial epithelial tissue was optimised using a curved dissection scissors and forceps. (A.). Image of tissue isolated from the functional layer of the endometrium using a curved dissection scissors and forceps. (B.). Comparison of tissue weight isolated using either a scissors or a curette for dissection.

Histology images taken before and after dissection (Figure 3.9) show that the uppermost functional layer containing the epithelium is being removed during dissection. In the intact endometrium (Figure 3.9 A.) we see the luminal epithelium forming a barrier overlying the stroma. The stroma also contains glandular epithelium and spiral arterioles which maintain the blood supply.
Figure 3.9. Representative histological image of endometrium before and after dissection of the epithelial layer for tissue culture.

(A). Histological image of the endometrium before dissection of the epithelial layer. Lum=endometrial lumen, LE=luminal epithelium, Str=stroma, GE=glandular epithelium. Scale bar indicates 200 μm. (B). Histological image of the endometrium following dissection of the epithelial layer (which is notably absent). Lum=endometrial lumen, Str=stroma, GE=glandular epithelium. Prior to and following dissection cross section of the endometrium were collected, formalin fixed and paraffin embedded before being sectioned into samples 4μm thick using a microtome. Sections were then mounted onto glass slides, de-paraffinised and stained with haemotoxylin before being imaged under an Olympus BX51 upright microscope.

Our initial protocol for cell isolation and transport involved collecting tracts at the abattoir and transporting them back on ice to the lab (transport time taking approximately 1-1.5 hours). However, cell viability using this approach was low. To improve cell viability, we performed dissections at the abattoir. Collected tracts were cleaned with 70% IMS and the exposed endometrium washed with PBS supplemented with 50 IU penicillin, 50 IU streptomycin and 2.5 µg/ml amphotericin B prior to dissection and the dissected tissue was transported back in culture media (RPMI supplemented with 10% FBS). This resulted in problems with bacterial contamination of our isolated cultures, probably due to the lack of a proper aseptic environment while performing the dissection in the abattoir. To address this issue, we investigated a number of different transport conditions. Tissue was transported back in media with or without FBS and with or without additional antibiotics. The tissue was also transported back on ice or at room temperature. Use of media lacking FBS but containing additional antibiotics and transported back at room temperature was found to result in high yields of viable cells.

Disruption of the harvested tissue was performed using both mechanical dissociation and by enzymatic dissociation. Mechanical dissociation was performed by using a sharp
scissors to chop the tissue finely (1-3 mm in size) before proceeding to enzymatic dissociation. An enzymatic digestion solution had previously been optimised containing trypsin (375 BAEE units). Collagenase (50 mg), BSA (100 mg) and DNase (10 mg) made up to 100 ml HBSS, as detailed in the original protocol published in 1988 and a more recent adaption (Fortier, Guilbault et al. 1988, Herath, Fischer et al. 2006). Low concentrations of trypsin were used to avoid damage to the cells. Discussion with others attempting to establish a similar model and previous reports in the literature had demonstrated that it was better to determine trypsin activity on activity units rather than volume due to the large batch to batch variation in the activity of the trypsin enzyme (MacKintosh, Schuberth et al. 2013). We found collagenase was able to digest the extracellular matrix, separating the epithelial and stromal cells but did not dissociate epithelial cells, allowing them to remain in viable clusters or islands. DNase was used to remove DNA released from lysed cells which can promote re-aggregation of dissociated cells. Incubation times ranging from 1-2 hours have been reported (Fortier, Guilbault et al. 1988, Cheng, Elmes et al. 2003, Herath, Fischer et al. 2006). We found that incubation at 37°C for 1 hour in a shaking incubator was sufficient for cell dissociation.

The initial protocol we used for isolation of pure populations of epithelial and stromal fibroblast cells involved adding the total cell isolate to a tissue culture flask and allowing 24 hours for stromal fibroblasts to adhere before the epithelial cells, still in suspension, were harvested and re-plated in a new flask (Herath, Fischer et al. 2006). We found that this did not lead to pure populations of either cell population and so looked for a new technique of isolating pure populations. Using the knowledge that epithelial cells are larger than stromal fibroblasts, we separated the populations using different size filters. Stromal fibroblasts easily pass through the 40 µm filters while the epithelial islands remain on the filter. Flipping the filters and backwashing the epithelial cells allows for the easy isolation of pure epithelial populations. Any residual stromal cell contamination of these epithelial populations can be easily removed by accutase based on the fact that stromal cells lift in the first 5 minutes of accutase treatment while epithelial cells take a minimum of 15 minutes to lift. Removal of red blood cells was performed by the addition of 1 ml sterile water to the cell pellet followed by gentle pipetting. Initial attempts at removing red blood cells included the use of red blood cell lysis buffer and vortexing for 30 secs. However, this resulted in a higher proportion of epithelial or stromal cell death.
More stromal fibroblast cells \((1.88 \times 10^6\) cells per uterine horn) than epithelial cells \((3.05 \times 10^5\) cell per uterine horn) were isolated following this process with >95% viability observed (Figure 3.10 A.).

Culture conditions were optimised beginning with coating the flasks to promote cell adherence. In the initial stages of optimising the primary culture protocol we found coating the tissue culture flasks with 0.1% gelatin helped to promote cell adherence. However, as we gained more competency in the primary culture protocol we found we no longer needed to coat the plates for the cells to adhere. We also examined the tissue culture growth media and its components. Using different amounts of FBS and trying supplements such as Insulin-Transferrin-Selenium (ITS-X) we found that the optimal growth media contained 10% FBS and 5% ITS. The ability of both cell populations to proliferate in culture is shown by the growth curve in Figure 3.10, with both cell populations maintaining a steady increase in cell numbers over the first 12 days of culture.

![Figure 3.10](image)

**Figure 3.10.** Higher yields of stromal fibroblasts are recovered during isolation and they demonstrate a more rapid expansion in culture compared to epithelial cells.

(A). Average epithelial cell yield isolated from tissue was \(3.08 \times 10^5 \pm 0.66 \times 10^5\), average stromal cell yield was \(1.88 \times 10^6 \pm 5.44 \times 10^5\) \((n=15)\). Following isolation cell pellets were re-suspended in 1ml of growth media and 10\(\mu\)l was diluted 1:1 with Trypan blue. Cells were counted under a light microscope using a haemocytometer. Values presented are mean cell counts per uterine horn ± SEM. (B). Growth curve showing the increase in cell populations over 12 days of culture. Endometrial epithelial (•) and stromal (○) cells were plated at a density of 9\(\times\)10\(^3\) cells into six-well tissue culture plates. Every four days, over the 12 days of culture, cells from one well of each plate was detached using trypsin, diluted in trypan blue and counted using a haemocytometer. Values presented are mean cell count ± SEM.

Purified cell populations were easily identified by morphology. Stromal fibroblasts could be found scattered throughout the flask (Figure 3.11 B. & D.) while epithelial cells grow
in small islands that ultimately combine to form a confluent monolayer (Figure 3.11 A. & C.).

Figure 3.11. Representative images of endometrial epithelial cells and stromal fibroblasts showing distinct morphological differences between the two cell populations.

Representative images of endometrial epithelial cells and stromal fibroblasts showing distinct morphological differences between the two cell populations. (A.). Small cluster of epithelial cells visible after 2 days of culture. (B.). Stromal fibroblasts appear scattered throughout the flasks following 2 days of culture. (C.). Pure population of endometrial epithelial cells following 14 days of culture. Scale bar indicates 100 μm. (D.). Pure population of endometrial stromal fibroblasts following 6 days of culture. Following the cell isolation protocol, cells were seeded in a T75 vented tissue culture flask and grown to confluence. Images were taken using an Olympus IX81 inverted microscope with camera attached. Scale bar indicates 100 μm.

We next investigated whether endometrial cells could be cryopreserved or passaged using standard protocols. The growth curve in Figure 3.12 A. shows that both cell populations recover well from cryopreservation and continue to proliferate in culture. No morphological differences were observed in the cryopreserved cells. Beyond two
passages morphological differences begin to appear in both cell populations and both populations become slower to proliferate, taking approximately 5-6 days to double their population as opposed to 2 days after just one passage (Figure 3.12 B.).

Figure 3.12. Proliferation of endometrial epithelial cells and stromal fibroblasts following cryopreservation and passaging.

(A). Growth curve showing the increase in cell populations over 12 days of culture. Endometrial epithelial (•) and stromal (◦) cells were plated at a density of 9x10^3 cells into six-well tissue culture plates. Every four days, over the 8 days of culture, cells from one well of each plate was detached using trypsin, diluted in trypan blue and counted using a haemocytometer. Values presented are mean cell count ± SEM. (B). Doubling time across consecutive passages of both endometrial epithelial (•) and stromal cells (◦). Both cell populations increase their doubling time following passaging. Cells were plated at a density of 9x10^3 cells into six-well tissue culture plates. Every four days, over the 16 days of culture, cells were detached using trypsin, diluted in trypan blue and counted using a haemocytometer before being reseeded into the 6 well plate at the same density. The doubling time value was obtained for each passage according to the formula DT = CT/CD, where CT represents the culture time and CD = log (Nc/No)/log2 represents the number of cell generations (Nc represents the number of cells at confluence, No represents the number of seeded cells).
Purity of the epithelial and stromal cells populations was demonstrated by the expression of cell specific cytoskeletal proteins. Epithelial cells are known to express cytokeratin while stromal cells are known to express vimentin (Franquemont, Frierson et al. 1991, Wonodirekso, Au et al. 1993). Examining the expression of the genes encoding these cytoskeletal proteins found that epithelial cells express higher levels of keratin-18 mRNA than vimentin mRNA while the opposite is true of stromal fibroblasts (Figure 3.13).

![Figure 3.13. Epithelial cells demonstrate higher levels of keratin-18 mRNA expression while stromal fibroblasts demonstrate higher vimentin mRNA gene expression.](image_url)

Endometrial cell populations were probed for the expression of genes encoding the cytoskeletal proteins keratin-18 and vimentin. (A.). 1.5×10⁵ epithelial cells or (B.), stromal fibroblasts were harvested in TRIzol. Total RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and keratin-18 (a marker of epithelial cells) and vimentin (a marker of stromal cells) gene Cq values were normalised to the reference gene H3F3A. Columns indicate mean relative mRNA gene expression, error bars indicate standard error of the mean (n=5).

Protein expression of the cytoskeletal proteins cytokeratin and vimentin was confirmed by immunofluorescent and immunocytochemical staining (Figure 3.14 and Figure 3.15). Epithelial cells can be observed staining positive for cytokeratin and negative for vimentin while the opposite is observed in stromal fibroblasts, which stain positive for vimentin and negative for cytokeratin.
Figure 3.14. Endometrial cell populations can be identified based on their differential expression of cytoskeletal proteins by immunofluorescence.

Immunofluorescent staining of endometrial epithelial cells and stromal fibroblasts with antibodies against the cytoskeletal proteins cytokeratin and vimentin. $1.5 \times 10^5$ endometrial epithelial cells or stromal fibroblasts were seeded in a 24 well plate and allowed to adhere overnight. Cells were then formalin fixed and stained with murine antibodies against human cytokeratin and vimentin cytoskeletal proteins, which are characteristic of epithelial and stromal cells respectively, followed by a fite-conjugated goat anti-mouse polyclonal secondary antibody. Dapi stain was applied to identify nuclei. An anti-mouse IgG control was included as a negative control. Cells were viewed under light, GFP fluorescence or Dapi fluorescence using an Olympus IX81 inverted microscope. Scale bar indicates 100 µm.
Figure 3.15. Endometrial cell populations can be identified based on their differential expression of cytoskeletal proteins by immunocytochemistry.

Immunocytochemical staining of endometrial epithelial cells and stromal fibroblasts with antibodies against the cytoskeletal proteins cytokeratin and vimentin. \(1.5 \times 10^5\) endometrial epithelial cells or stromal fibroblasts were seeded into wells of the Millicell EZ SLIDE 8 well glass culture slides and allowed to adhere overnight. Cells were then fixed in ice cold methanol before blocking in 3% \(\text{H}_2\text{O}_2\) for 20 mins followed by blocking in 10% BSA for 1 hour. Cells were incubated overnight with antibodies against human cytokeratin and vimentin cytoskeletal proteins. Cells were then washed in PBS before incubation with the secondary antibody for 30 mins followed by incubation with the chromagen DAB for 10 mins. Slides were mounted using Surgipath mounting medium before being examined using an Olympus BX51 upright microscope. Scale bar indicates 100 \(\mu\)m.
The expression of the cytoskeletal proteins by the distinct endometrial cell populations as observed by immunofluorescent and immunocytochemical staining was confirmed by Western blotting for cytokeratin in epithelial cells (Figure 3.16 A.) and vimentin in stromal fibroblasts (Figure 3.16 B.).

**Figure 3.16.** Endometrial cell populations can be identified based on their differential expression of cytoskeletal proteins by Western blotting.

Endometrial (A.) epithelial cells or (B.) stromal fibroblasts were harvested in RIPA lysis buffer. Total protein levels were measured using a BCA assay and 20 µg of protein was loaded on an SDS-PAGE gel. Gel electrophoresis was performed and the resulting gel was electrophoretically transferred onto a PVDF membrane before incubation in blocking buffer (5% milk powder in PBS-T) for 1 hour followed by incubation overnight at 4°C with an antibody directed against either cytokeratin, vimentin or β-actin (loading control). The membrane was washed three times in PBS-T before incubation for 1 hour at room temperature with the appropriate secondary antibody conjugated to HRP. The membrane was washed a further three times before visualisation using the Biorad ChemiDoc MP system and HRP substrate solution (ECL).

As we aim to use our primary culture model to investigate the innate immune responses of the cells we needed to be certain that there was no immune cell contamination of our cultures which might give a false-positive result. A PCR assay for *PTPRC*, the gene that encodes CD45, a marker of immune cells was optimised. PBMC cDNA was used as a positive control. The housekeeping gene *RPS9* was included to ensure cDNA quality was adequate. Cultures were negative for the presence of CD45 (Figure 3.17) indicating an absence of immune cell contamination, again confirming the purity of the endometrial cell populations.
Figure 3.17. Distinct endometrial cell populations lack immune cell contamination.

Endometrial epithelial cell and stromal fibroblast populations were probed for the expression of PTPRC. Total RNA was extracted from $1.5 \times 10^5$ endometrial epithelial cells (A.) and stromal fibroblasts (B.) and first stand cDNA reverse transcribed. PCR was carried out for PTPRC and RPS9 and following PCR amplification 2% agarose gel electrophoresis confirmed amplicons of the expected size. PBMC cDNA was used as a positive control for PTPRC amplification. A non-template control from which DNA was omitted was included to detect any nucleic acid contamination. PTPRC amplicon size=845 bp; RPS9 amplicon size=270 bp.

Having generated pure endometrial cell populations, a time course of the response of these cells to the microbial PAMP, LPS, was performed to investigate their ability to detect and respond to inflammatory stimuli compared to the more classically defined immune cells present in PBMCs. Results indicate that epithelial and stromal fibroblast populations display significantly divergent response profiles (Figure 3.18).

IL8 mRNA levels, as quantified using qPCR, was constitutively expressed across epithelial cells ($4.3 \times 10^{-1}$ relative expression units to $H3F3A$), stromal fibroblasts ($2.8 \times 10^{-1}$) and unmatched PBMCs ($3.1 \times 10^{-1}$) (Figure 3.18 A.). IL8 mRNA induction peaked at 3 hours post-stimulation in epithelial cells with a MFC of 217.0 while stromal fibroblasts showed peak induction of IL8 at 24 hours post-stimulation with a MFC of 40.9, similar
to PBMCs which demonstrate peak IL8 mRNA production at 12 hours post stimulation with a MFC of 14.7 (Figure 3.18 B.-D.).

Constitutive expression of IL6 mRNA was detected in epithelial cells (1.9×10^{-1} relative expression units), PBMCs (0.3×10^{-1}) and stromal fibroblasts (0.5×10^{-1}) (Figure 3.18 E.). Induction of IL6 mRNA demonstrated a similar pattern to IL8, with peak production of IL6 occurring in epithelial cells at 3 hours post stimulation (MFC of 41.4; p=0.0230), with induction of IL6 mRNA by stromal fibroblasts (MFC of 14.9) and PBMCs (MFC of 6.9) occurring at the same time-point (Figure 3.18 F.-H.).

In contrast, constitutive expression of the antimicrobial genes S100A8 and S100A9 was significantly higher in PBMCs compared to the low levels detected in endometrial epithelial cells (p<0.0001) and stromal fibroblasts (p=0.0004) (Figure 3.18 I. & M.). However, upon stimulation with LPS endometrial cells were more responsive compared to PBMCs, with epithelial cells demonstrating stronger induction of both S100A8 (MFC of 55.6 in epithelial cells at 6 hours post stimulation compared to a MFC of 0.7 in PBMCs and a MFC of 4.0 in stromal fibroblasts) (Figure 3.18 J.-L.) and S100A9 (MFC of 57.8 in epithelial cells at 24 hours post stimulation compared to a MFC of 1.5 in PBMCs and a MFC of 6.7 in stromal fibroblasts) (Figure 3.18 N.-P.).
Figure 3.18. Endometrial epithelial cells and stromal fibroblasts express *IL6, IL8, S100A8* and *S100A9* mRNA in response to LPS stimulation.

(A.-D.) *IL8*, (E.-H.) *IL6*, (I.-L.) *S100A8* and (M.-P.) *S100A9* mRNA levels in PBMCs, epithelial cells and stromal fibroblasts following a time course stimulation with LPS (2 µg/ml). Following stimulation cells were harvested in TRIzol reagent, total RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene H3F3A. Columns indicate either mean relative expression to H3F3A or mean fold change relative to unstimulated control. Error bars indicate standard error of the mean, (n=3). Statistical analysis was performed using either a Kruskall Wallis test with Dunns multiple comparison test or using a Mann Whitney test, comparing untreated control to a time point stimulation. **=p≤0.01; ***=p≤0.001; ****=p≤0.0001.
3.3.4 Development of polarized epithelial cell model

One limitation of studying epithelial cell responses in 2D cultures is the fact that \textit{in-vivo} epithelial cells have a 3D morphology with a distinct apical versus basolateral polarity which is maintained by the tight junctions between epithelial cells (Wira, Grant-Tschudy et al. 2005). This allows them to differentially respond to antigens presented at either membrane and to orientate the secretion of pro- or anti-inflammatory molecules. To mimic this property \textit{in-vitro} we aimed to establish a polarized epithelial cell model by growing epithelial cells on hanging inserts which allows them to polarize and allows us to stimulate the cells from either the basolateral or apical membrane.

A number of studies in human, porcine and bovine have investigated the optimal conditions for the growth of epithelial cells on transwell inserts. Work by MacKintosh et al (2013) investigated the density of matrigel used to coat the transwell inserts to support the epithelial cells and the optimal brand of transwell insert used. They found Millipore™ transwell inserts to be optimal when coated with a thin layer of matrigel diluted 1:8 with media. We based our protocol around these findings and verified that they were the optimal conditions for establishment of the polarized epithelial cultures. Polarized epithelial cells (stained with haemotoxylin) can be observed growing on a transwell insert in \textbf{Figure 3.19 A}.

We examined confluency/integrity of the epithelial barrier using a FITC-dextran permeability assay which measures the trans-epithelial flux of a 4 kDa FITC labelled dextran molecule. The FITC-dextran is applied to the apical compartment of the transwell plate and 3 hours later the media is collected from the basolateral compartment and fluorescent activity is measured. As the cultures approach confluency and barrier integrity is achieved the amount of FITC-dextran moving through the transwell insert reduces to nothing. It took approximately 14 days for the epithelial cells to reach confluency and polarize, as measured by the FITC-dextran assay (\textbf{Figure 3.19 B}). Epithelial barrier integrity was further examined by measuring the trans-epithelial resistance (TER) (\textbf{Figure 3.19 C}). TER is a measurement of the electrical impedance provided by both the transcellular impedance of the apical and basolateral membranes of the epithelial cells in addition to the paracellular impedance provided by the epithelial tight junctions. As the epithelial barrier approaches confluency and the tight junctions between cells establish, the TER values will increase. The TER measurements support
the results the FITC-dextran assay, with epithelial barrier confluency reached after 14 days in culture, as indicated by a TER value >3 LogΩcm², (Figure 3.19 C.). TER cut-off values are based on previously reported values in the literature (MacKintosh, Schuberth et al. 2013, Srinivasan, Kolli et al. 2015).

Figure 3.19. Establishment of a model of polarized endometrial epithelial cells.

(A). Haemotoxylin staining of polarized epithelial cells growing on a transwell insert. Transwell inserts were formalin fixed and paraffin embedded before being sectioned into samples 4µm thick using a microtome. Sections were then mounted onto glass slides, de-parrafinised and stained with haemotoxylin before being imaged under an Olympus BX51 upright microscope. (B). Permeability of the polarized membrane was quantified by measuring the trans-epithelial flux of a 4-kDa FITC-labelled dextran molecule. Briefly, 200 µg of FITC-dextran was added to the apical surface of the monolayer. Following incubation at 37°C for 3 hours, a 200 µl aliquot of media from the basolateral compartment was analysed for FITC fluorescence. Values are expressed as a mean percentage of the no cell control ± SEM (n=5). (C.). Trans-epithelial resistance (TER) was used to examine the permeability of the epithelial barrier. TER values were recorded using an epithelial volt-ohm meter. Values were corrected for the area of the transwell insert and baseline levels were accounted for by measuring a blank (containing no cells) insert. A value greater than 3 logΩcm² indicated full polarization and confluency of the epithelial barrier.

Based on previous reports in the literature which reported that IL-6 was predominantly secreted apically while IL-8 could be secreted either apically or basolaterally by polarized endometrial epithelial cells (Healy, Cronin et al. 2015). This paper also reported that both
IL-8 and IL-6 expression was predominantly induced by stimulation at the apical membrane. We aimed to investigate whether our polarized epithelial cultures mirrored these results by measuring the mRNA induction of both IL8 and IL6 when the polarized epithelial cells were stimulated apically or basolaterally over a time course of 3, 6, 12 and 24 hours. Epithelial cells were grown to confluence on transwell inserts coated with a thin layer of matrigel and allowed to polarize over the course of 2 weeks. The polarized epithelial cells were then stimulated either apically or basolaterally with 2.0 µg/ml LPS for 3, 6, 12 and 24 hours.

Induction of IL6 mRNA peaked at 6 hours post-stimulation in both apically and basolaterally stimulated cells, with a MFC of 25.9 in the apical stimulations compared to a MFC of 3.7 in the basolateral stimulations (Figure 3.20 A.). Induction of IL6 was reduced to a MFC of 1.5 in the apical stimulations at 24 hours post-stimulation compared to a MFC of 0.7 in the basolateral stimulations at the same time-point. IL8 mRNA induction peaked at 6 hours post-stimulation with a MFC of 58.4 in the apically treated epithelial cells compared to a MFC of 9.9 in the basolaterally treated cells (Figure 3.20 B.).

These results confirm that our polarized epithelial cell model responds differentially to stimuli presented at either membrane and that this response is in line with what has previously been observed in the literature.
**A.** IL6 mRNA Expression in Polarized Epithelial Cells

![IL6 mRNA Expression Graph](image)

**B.** IL8 mRNA Expression in Polarized Epithelial Cells

![IL8 mRNA Expression Graph](image)

Figure 3.20. *IL6* and *IL8* mRNA are induced following apical stimulation in polarized epithelial cells.

(A). *IL6* and (B). *IL8* mRNA fold change following stimulation. Epithelial cells were grown to confluency on transwell inserts coated with matrigel and allowed to polarize before stimulation with LPS in either the apical or basolateral compartment. Following stimulation, the cells were harvested in TRIzol. RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene *H3F3A*. Columns indicate mean fold change relative to unstimulated control, error bars indicate standard error of the mean (*n*=4).
3.4 Discussion

Infection of the endometrium by microbial and viral pathogens can result in pathological inflammation that is harmful to cow health and agricultural sustainability (Sheldon, Williams et al. 2008, Bromfield, Santos et al. 2015). Epithelial and stromal fibroblast cell populations play important but distinct roles in innate defence of the endometrium. Understanding the inflammatory pathways and mechanisms of response in endometrial cells is crucial to developing therapeutics targeted towards the specific inflammatory insult, thereby improving cow health and fertility and ultimately increasing profitability. Elucidating these inflammatory pathways requires a reliable and well characterised model in order to determine which cell type is contributing to the inflammation.

Investigation into production of inflammatory mediators and inflammatory signalling pathways within the bovine endometrium is limited by the research models available. While a number of options exist, each possesses its own unique advantages and caveats that impacts its potential to mirror what is occurring in-vivo. We investigated the reliability of a number of different models of endometrial cells, including the BEND cell line, endometrial explants and the primary culture of endometrial epithelial and stromal fibroblasts.

The bovine endometrial epithelial cell line (BEND cells) represent a cost effective and unlimited supply of endometrial epithelial cells and provided a strong basis for investigating inflammatory pathways within the endometrium. However, with observable differences in morphology across passages, with cells visibly becoming more fibroblast-like in appearance, and the increased expression of the gene encoding the fibroblast cytoskeletal protein vimentin, it meant that BEND cells were an unreliable model for further investigations. In addition, working with BEND cells meant we were limiting our investigation to only epithelial cell mediated inflammatory responses. With roles for fibroblasts in mediating inflammatory responses emerging in pathological inflammatory conditions such as rheumatoid arthritis, any model investigating endometrial inflammation must take their contribution into account (Mizoguchi and Slowikowski 2018). The unreliability of the BEND cell line and the lack of any other commercially available model has greatly hindered research into bovine endometrial immune responses to date.
Endometrial explants provide the closest approach to modelling in-vivo conditions, allowing for stimulations of cell populations in their correct spatial orientation and respective ratios and have previously been demonstrated for use in modelling bovine endometrial inflammatory responses (Borges, Healey et al. 2012, Noleto, Saut et al. 2017). Tissue composition and communication between cells is maintained while also allowing for the inclusion of endothelial cells and macrophages, in addition to the epithelium and stromal fibroblasts, providing a potential link between cellular studies and in-vivo models. However, explants are greatly restricted by their limited viability in-vitro and this has meant that only shorter time frame stimulations may be performed, with a previous report using human endometrial explants recommended culturing explants for a maximum of 6 hours (Schäfer, Fischer et al. 2011). While we found that explants were able to maintain their normal tissue architecture in culture, examining the baseline expression levels of a number of inflammatory cytokines revealed explants exist in a heightened inflammatory state, possibly due to the presence of DAMPs within the tissue, generated by the tissue dissection released. This limits their usefulness in investigating cell specific responses to inflammatory stimuli, which is evidenced in their non-existent fold induction of cytokine mRNA levels, possibly due to the already heightened mRNA expression of cytokines within the explants. Based on these observations, we concluded that explants of limited value in examining immune responses.

The isolation of pure populations of primary endometrial epithelial cells and stromal fibroblasts allows for the examination of cell specific responses and signalling pathways operating within the distinct endometrial cell populations. Bovine endometrial epithelial cells and stromal fibroblasts have been grown with varying degrees of success since the first reports describing their isolation (Fortier, Guibault et al. 1988, Herath, Fischer et al. 2006). As primary cells of different tissues and indeed species differ significantly in their culture requirements, we optimised a number of different conditions, including isolation, transport, culture, their ability to be passaged and cryopreserved, in addition to thoroughly characterising the isolated cell populations to ensure cell purity. We found that our optimised protocol gave consistently pure and viable populations of epithelial and stromal cells, confirmed by the positive staining for their respective cytoskeletal proteins cytokeratin and vimentin and by the easily observed differences in cell morphology. We also confirmed the absence of immune cell contamination by PCR for PTPRC which encodes CD45, the immune cell surface marker, to ensure that any
upregulation in immune gene expression was due to the endometrial cell populations and not contaminating immune cells. While we were able to collect breed and age details on a large number of the cows sampled in this study, we had insufficient numbers to identify breed specific differences, which could possibly account for some of the observed variation in our assays.

Our optimised protocol contains several key modifications to previously published protocols. Dissection and harvesting of the endometrium occurs immediately after recovering the tract in the abattoir as opposed to transporting the tract back to the laboratory on ice. We found this greatly improved cell viability and numbers of cells recovered. Separating cells based on size using differential filtering also greatly improved the purity of the distinct cell populations. We found the brand of cell strainer used impacted on cell recovery. In addition, any residual stromal cell contamination of our epithelial cultures was removed with accutase treatment based on differential lifting time. Finally, supplementing the growth media with insulin, transferrin, selenium solution (ITS-X) also had a marked impact on the growth of our cell populations in culture.

The induction of inflammatory cytokine mRNA in response to LPS stimulation confirms that isolated and cultured endometrial cells retain the ability to respond to bacterial stimuli in-vitro. While the induction of the cytokines IL8 and IL6 by endometrial cells has been previously reported (Cronin, Turner et al. 2012, Healy, Cronin et al. 2015), their induction here, in addition to the antimicrobial S100A8 and S100A9, confirms that our improved protocol for isolating and culturing endometrial cells provides a practical option for the study of bovine inflammatory responses in-vitro. The induction of these inflammatory mediators also revealed endometrial cell populations demonstrate divergent levels of induction, indicating that each cell population analyses possesses a distinct inflammatory profile. This finding has potential implications for our future investigations into IL-1β induction by these endometrial cells.

The establishment of a polarized model of epithelial cells allows for investigations into the specialised properties of epithelial cells such as their ability to directionally secrete proteins from either their apical or basolateral membrane in addition to studying their ability to form tight junctions with neighbouring cells and establish an epithelial barrier (Glasser, Julian et al. 1988). The polarized epithelial cell model also allows for the spatial introduction of immunostimulatory factors, allowing us to mirror what is occurring in the
Postpartum period *in-vivo* with disruption to the epithelial barrier caused by the process of parturition. Our work here confirmed the ability of bovine endometrial epithelial cells to form a confluent, intact epithelial barrier when cultured *in-vitro* transwell inserts. Our results also demonstrated that the inflammatory cytokines *IL6* and *IL8* are preferentially induced through apical stimulation, confirming what had been observed in previous studies (MacKintosh, Schuberth et al. 2013, Healy, Cronin et al. 2015). The ability of epithelial cells to form an intact barrier *in-vitro* will also allow for future investigations into the effect of inflammatory cytokines, such as IL-1β, on barrier disruption and function. The development of a calcium-switch assay allows for the depletion of calcium from *in-vitro* cultures through the addition of EDTA, a calcium chelating agent (Gumbiner 1986, Glover, Bowers et al. 2013). This interferes with the epithelial tight junctions and causes complete disruption to the epithelial barrier. The removal of EDTA allows for the re-establishment of the epithelial barrier in real-time, similar to postpartum tissue repair events. Inflammatory cytokines or other stimuli can be added to the cultures at this time and their effect on barrier re-formation examined.

This primary culture model of bovine endometrial epithelial cells and stromal fibroblasts represents a practical tool for veterinary science research and will facilitate investigations into the production of immune molecules such as cytokines, chemokines, HDPs and acute phase proteins under defined inflammatory conditions. This model will ultimately allow us to address our central hypothesis, investigating the production and regulation of IL-1β locally within the endometrium by the both epithelial cells and stromal fibroblasts.
Chapter 4 IL-1β induction and expression in the endometrium

4.1 Introduction

The IL-1 family exhibits potent pro-inflammatory properties at sites of tissue infection or damage, acting on immune cells to drive their function and influence their survival. Since the initial report of the IL-1β cDNA sequence in 1984 (Auron, Webb et al. 1984, Lomedico, Gubler et al. 1984), the IL-1 family has grown to encompass the cytokines IL-1α, IL-1β, IL-18, IL-33, IL-1RA and IL-1F5 to IL-1F10 (Taylor, Renshaw et al. 2002). In the human, all IL-1 family members are located within a 400kb stretch on chromosome 2, with the exception of IL-18 and IL-33 (located on chromosomes 11 and 9 respectively). In mice, the IL-1 family is located on chromosome 2 with the exception of IL-18 which is located on chromosome 9 and IL-33 which is located on chromosome 19. In the bovine, IL-1 family members are located on chromosome 11 with IL-18 located on chromosome 15 and IL-33 located on chromosome 8. IL-1α and IL-1β both possess conserved gene structures with each gene consisting of 7 exons (UCSC 2017).

Most IL-1 gene family members are translated as inactive proteins containing a pro-domain that must be cleaved for cytokine release from the cell. Cleavage of these cytokines from their pro-form results in a conformational change in the protein structure, which is important for receptor binding and subsequent biological activity (Hazuda, Strickler et al. 1991). Cleavage into a mature active cytokine is mediated by the inflammasome complex (Martinon, Burns et al. 2002). All family members apart from IL-1RA lack a signal peptide meaning that their release from the cell is not through the conventional ER-Golgi secretion pathway. A number of different secretion mechanisms have been proposed to account for the fact that these cytokines do not utilise the classical ER-Golgi route of secretion. These include the release of these cytokines through secretory lysosomes, microvesicle shedding, exosome release, secretory autophagy, passive release during cell lysis (pyroptosis) and plasma membrane translocation. Despite intensive research there is still not sufficient evidence to support one or more pathways in mediating IL-1 release from the cell (Monteleone, Stow et al. 2015).

Despite possessing similarities in gene structure and the requirement for processing by the inflammasome, IL-1 family members exhibit many contrasting functions. IL-1α and IL-1β both act on the IL-1R which drives activation and increased phagocytosis in
macrophages, increased oxidative burst in neutrophils and increased cytokine production and up-regulation of MHC molecules in dendritic cells (Dinarello 2009). IL-1α is more restricted to acting locally in the environment in which it is produced while IL-1β has been shown to act systemically (Kurt-Jones, Beller et al. 1985). IL-18 is constitutively expressed but must be cleaved from its pro-form into its active form in order to be released from the cell. IL-33, like IL-1α, does not need processing for its activity, however full length IL-33 results in lower activity than processed IL-33 upon binding to its receptor, (Talabot-Ayer, Lamacchia et al. 2009). In addition to the effects mentioned above for IL-1α and IL-1β, IL-18 and IL-33 have also been shown to drive the production of IFN-γ in NK and NKT cells (Sims and Smith 2010).

IL-1β protein is expressed in human, mouse and bovine reproductive tracts, although the role it plays here has yet to be fully elucidated. Expression of IL-1β and its receptor IL-1R in the bovine endometrium have been localised in the luminal and glandular epithelium by immunohistochemistry (Correia-Alvarez, Gomez et al. 2015). Expression of IL-1R has also been demonstrated on individual endometrial epithelial and stromal fibroblast populations in response to bacterial stimulation (Healy, Cronin et al. 2014). IL-1β also plays roles in implantation with reports confirming that blastocysts secrete and respond to IL-1β and that the concentration of IL-1β in embryo conditioned media positively correlated with successful implantation (Baranao, Piazza et al. 1997).

In the human IL-1β production has been associated with many pathologies (such as rheumatoid arthritis), driving cell death and inhibiting tissue repair and cell proliferation. IL-1β has been strongly linked to driving pancreatic β-cell death in type-1 diabetes (Gurzov, Ortis et al. 2009). IL-1β has also been shown to mediate cell cycle arrest and drive apoptosis in neural precursor cells resulting in neurodegenerative disease and stroke (Guadagno, Swan et al. 2015). Given the role IL-1β plays in driving inflammation and ultimately cell death in these inflammatory pathologies, we began to question its role in postpartum endometritis.

Our group has previously described raised levels of IL1B mRNA expression in endometritic cows at 7 DPP, which remained elevated at 21 DPP (Foley, Chapwanya et al. 2015). Other IL-1 family members including IL1A and IL1RI were differentially expressed between 7 and 21 DPP in healthy cows while the IL-1 receptor subunits IL1RI and IL1R2 along with IL18, IL18RAP and IL1RAP were differentially expressed between
healthy and endometritic cows at 21DPP. Research in the bovine is often restricted to examining mRNA as reagents for protein studies are limited. Here we aimed to validate IL-1β expression at the protein level using protein isolated from endometrial tissue collected from healthy or endometritic cows at two time-points postpartum.

Our previous study used endometrial biopsies which are mixed cell populations containing epithelial cells, stromal cells and invading immune cell populations present in the endometrium at the time of sample collection. Using a reductionist approach and examining induction of IL-1 family members in primary epithelial, stromal and PBMCs we aimed to elucidate the cellular source and the conditions required for production of IL-1 in the endometrium. Classical producers of the IL-1 family of cytokines include monocytes, macrophages and dendritic cells. However there is growing awareness in the literature that previously ignored cell populations such as the epithelial and stromal cells may play a role in production of IL-1 family members particularly at mucosal sites such as the lungs, intestine and FRT. Human and murine intestinal epithelial cells have been shown to produce inflammasome mediated IL-18 (Knodler, Crowley et al. 2014) and there is a growing appreciation for the role of stromal cells in driving inflammatory responses (Nowarski, Jackson et al. 2017). Identifying the principal cell population responsible for producing IL-1β in the bovine endometrium will allow us to further elucidate the mechanisms behind its production and determine its role in driving pathological inflammation in the post-partum bovine endometrium.
4.2 Hypothesis and specific aims

We hypothesize that endometrial epithelial and stromal fibroblast cells produce IL-1β locally, resulting in elevated IL-1β protein levels in the postpartum endometrium which drive the pathological inflammatory immune response associated with postpartum endometritis.

The specific aims were:

1. Examine IL-1β protein levels in endometrial tissue collected at 7 and 21 DPP from healthy and endometritic cows.
2. Examine the constitutive mRNA expression and subsequent induction of IL-1 family members and IL-1β protein induction in endometrial epithelial and stromal fibroblast cells and compare this to IL-1β production in PBMCs.
3. Examine the directional induction and secretion of IL-1β in a model of polarized endometrial epithelial cells.
4. Examine the effect of pathological concentrations of IL-1β on epithelial barrier function.
4.3 Results

4.3.1 IL-1β protein levels are elevated in endometrial samples from endometritic cows

Having previously observed that *IL1B* mRNA levels were significantly elevated in our mRNA-seq dataset, we aimed to investigate if this result was reflected at the protein level given the role of IL-1β in inflammasome mediated inflammation. Due to technical and ethical issues surrounding the collection of endometrial biopsies postpartum, we aimed to investigate the use of endometrial samples collected using cytobrushes as a means to assess the inflammatory environment of the postpartum endometrium. In recent years, endometrial sample collection using cytobrushes has emerged as one of the standard diagnostic techniques for studying bovine endometritis as they allow for a more consistent and less invasive method of harvesting a representative population of cells from the endometrium (Barlund, Carruthers et al. 2008).

As part of a larger investigation, endometrial samples were collected at two time-points postpartum. Cows were retrospectively classified as being either healthy or having cytological (or sub-clinical) endometritis (CE) or cytological endometritis with PVD (the clinical form of disease) (CE+PVD) based on immune cell infiltrate as observed by cytology slides prepared from the endometrial cytobrushes. Clinical presentation of disease was classified by observing and scoring vaginal discharge using previously reported criteria (Williams, Fischer et al. 2005). Cows were retrospectively divided into groups based on the immune cell infiltrate at 21 days postpartum, with those having low levels of neutrophils (considered <18% of cells present) at 21 DPP considered healthy while those cows with high neutrophils (considered >18% of cells present) at 21 DPP considered to have CE. Cattle with clinical endometritis (CE+PVD) were diagnosed based on mucus scoring.

Animal classifications are detailed in Table 4.1. Neutrophil counts in the healthy cows reduce in number, from 31.6% 7 DPP to 8.0% 21 DPP, indicating the ability of healthy cows to resolve their postpartum inflammation in a timely manner. In cows with CE or CE+PVD, neutrophil counts increase from 7 DPP to 21 DPP (55.5% 7 DPP to 74.2% 21 DPP in cows with CE and 52.8% 7 DPP to 83.3% 21DPP in cows with CE+PVD), indicating exacerbation of the inflammatory response. The length of the calving to
conception interval, recorded here as ‘Days Open’, was also increased in cows with uterine disease (92.6 days in healthy cows vs. 94.7 in cows with CE and 106.8 in cows with CE+PVD), demonstrating the effect this pathological inflammatory event is having on the resumption of normal fertility.

Table 4.1. Details of classification of endometrial samples obtained by uterine cytobrush.

Cows were retrospectively classified as healthy or as suffering from uterine disease following endometrial sampling using a uterine cytobrush. Neutrophil counts were assessed by cytology scoring of uterine cytobrush rolled out onto a glass slide. Vaginal mucus scoring (VMS) was assessed using previously published criteria (Williams, Fischer et al. 2005). The average days open, representing the calving to conception rate, and the cow’s previous number of lactations were recorded by the farmer.

<table>
<thead>
<tr>
<th></th>
<th>Healthy (n=7)</th>
<th>Cytological Endometritis (n=7)</th>
<th>Cytological Endometritis with PVD (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Neutrophils 7DPP</td>
<td>31.6 (Range: 2.0-84.5)</td>
<td>55.5 (Range: 31.0-78.5)</td>
<td>52.8 (Range: 23.5-74.5)</td>
</tr>
<tr>
<td>% Neutrophils 21DPP</td>
<td>8.0 (Range: 2.5-15.5)</td>
<td>74.2 (Range: 60.5-93.0)</td>
<td>83.3 (Range: 65.5-93.5)</td>
</tr>
<tr>
<td>7 DPP VMS Range</td>
<td>0-1</td>
<td>0-5</td>
<td>0-2</td>
</tr>
<tr>
<td>21 DPP VMS Range</td>
<td>0-1</td>
<td>0-2</td>
<td>4-5</td>
</tr>
<tr>
<td>Average Days Open</td>
<td>92.6 (Range: 72-131)</td>
<td>94.7 (Range: 54-114)</td>
<td>106.8 (Range: 77-162)</td>
</tr>
<tr>
<td>Average Number of Lactations</td>
<td>3.7 (Range: 1-7)</td>
<td>5.1 (Range: 1-10)</td>
<td>3.7 (Range: 1-6)</td>
</tr>
</tbody>
</table>
TRIzol reagent was used to process the endometrial cytobrush samples as this allows for dual extraction of protein and RNA. TRIzol was also preferable over normal protein lysis buffers due to its strong corrosive properties which allows it to easily disrupt the mucus in some of the cytobrush samples. Following protein extraction, protein yields were quantified using a BCA assay and were in the range of 1.2 mg/ml ± 1.1 (Figure 4.1). Clear differences in protein yields were visible between the different disease classifications. At 7 DPP cytobrushes from cows with cytological endometritis yielded the highest level of total protein (2.5 mg/ml versus 0.3 mg/ml in healthy cows versus 1.0 mg/ml in cows with CE+PVD). The differences between groups are particularly striking at 21 DPP with a clear decrease in protein yield observed as disease severity increases (1.9 mg/ml in healthy cows versus 1.0 mg/ml in cows with CE and 0.6 mg/ml in cows with CE+PVD).

![Protein Yields](image)

**Figure 4.1 Protein yields differ between disease classifications at 7 and 21 DPP.**

Total protein levels were quantified in endometrial samples obtained by cytobrush at 7 and 21 DPP. Following collection samples were stored at -80°C until protein extraction using the TRIzol method. Total protein was quantified using a BCA assay. Cows were retrospectively classified as being healthy or having endometritis based on cytology scoring of infiltrating neutrophils. Columns indicate mean protein concentration per mg of total protein (TP), error bars indicate standard error of the mean (7 DPP: healthy n=5, cytological endometritis n=5, cytological endometritis with PVD n=3; 21 DPP: healthy n=5, cytological endometritis n=3, cytological endometritis with PVD n=7).

Quantification of IL-1β levels by ELISA demonstrated a higher level of IL-1β expression in cows with endometritis at both 7 and 21 DPP (Figure 4.2). At 7 DPP a clear gradient
in IL-1β expression is evident with the disease grades. As disease severity increases, the levels of IL-1β increase concurrently. IL-1β levels in cows with CE+PVD are significantly higher than healthy cows (4467.12 pg/mg TP versus 635.6 pg/mg TP, p=0.04) and while not significant, IL-1β levels in cows with CE was higher than healthy cows (2014.3 pg/mg TP versus 635.6 pg/mg TP). At 21 DPP there was a clear distinction in IL-1β levels between cows with CE+PVD versus healthy cows (5693.8 pg/mg TP versus 644.1 pg/mg TP, p=0.01). IL-1β levels in cows with CE alone were comparable to healthy cows (787.2 pg/mg TP versus 644.1 pg/mg TP).

**Figure 4.2 IL-1β protein levels are elevated in endometritic cows at 7 and 21 DPP.**

IL-1β protein levels were quantified in endometrial cytobrushes collected at 7 and 21 DPP. Following collection samples were stored at -80°C until protein extraction using the TRIZol method. Total protein was quantified using a BCA assay and a commercially available bovine specific IL-1β ELSA kit was used to quantify IL-1β levels. Cows were retrospectively classified as being healthy or having endometritis based on cytology scoring of infiltrating neutrophils. Clinical disease was diagnosed by scoring vaginal discharge. Columns indicate mean protein concentration per mg of total protein (TP), error bars indicate standard error of the mean (7 DPP: healthy n=5, cytological endometritis n=5, cytological endometritis with PVD n=3; 21 DPP: healthy n=5, cytological endometritis n=3, cytological endometritis with PVD n=7). Statistical analysis was performed using a Mann Whitney test between healthy and individual endometritic groups at each time-point, *p≤0.05.

Total protein extracted from the cytobrush endometrial samples will contain both secreted proteins and proteins retained within cells and so will most likely contain both pro- and active IL-1β. As the ELISA cannot distinguish between the two forms, we set out to confirm the presence of the active form by Western blotting (Figure 4.3). Western
blotting confirmed the presence of the active 17 kDa secreted form of IL-1β and the 35 kDa pro-form. Additionally, we also observed two other forms of IL-1β in our endometrial sample lysates. An isoform which we labelled as a high molecular weight active-IL-1β was identified at approximately 26 kDa in size and a high molecular weight pro-IL-1β was identified at approximately 55 kDa in size. The presence of these isoforms was confirmed by the use of two bovine specific IL-1β antibodies purchased from two different companies. Similar IL-1β isoforms were observed in our cell culture stimulations and our investigation into these isoforms is examined in section 4.7.

![Image](image_url)

**Figure 4.3. Novel IL-1β isoforms are present in protein extracted from endometrial samples postpartum.**

Following protein extraction using the TRIzol method, endometrial protein samples were probed for IL-1β expression by western blotting. 20 μg of sample was loaded on an SDS-PAGE gel. Gel electrophoresis was performed and the resulting gel was electrophoretically transferred onto a PVDF membrane before incubation in blocking buffer (5% milk powder in PBS-T) for 1 hour followed by overnight incubation at 4°C with an antibody directed against bovine IL-1β. The following day the membrane was washed three times in PBS-T before incubation for 1 hour at room temperature with a secondary antibody conjugated to HRP. The membrane was then washed a further three times before visualisation using the Biorad ChemiDoc MP system and HRP substrate solution Active and pro-IL-1β are located at 17 kDa and 35 kDa respectively. The higher M.W. active and pro-IL-1β are located at 26 kDa and 55 kDa respectively.

Using the RNA extracted from these endometrial samples we examined the expression of the main inflammasome receptors associated with IL-1β production (**Figure 4.4**). NLRP3 demonstrated the highest level of expression and so became the focus of our subsequent investigations into IL-1β regulation in the endometrium (**Figure 4.4 A**). Levels of NLRP3 were higher in cows with CE+PVD versus healthy at both time points (0.5 relative expression units to H3F3A versus 0.05 7 DPP, 6.6 relative expression units
versus 0.5 21 DPP). Low levels of IFI16 and NLRC4 were detected (Figure 4.4 B.-C.). Expression of IFI16 was higher in endometritic cows 21 DPP (0.2 units in cows with CE+PVD and 0.1 units in cows with CE alone versus 0.02 units in healthy cows) (Figure 4.4 B.). Levels of NLRC4 were higher in healthy cows 21 DPP compared to cows with CE+PVD (0.12 units versus 0.07 units) (Figure 4.4 C.). No expression of NLRP1 was detected (Figure 4.4 D.). The AIM2 inflammasome receptor has previously been reported to be pseudogenised in the bovine, rendering it non-functional and so its expression was not examined here (Cridland, Curley et al. 2012).

Figure 4.4 Inflammasome receptor mRNA expression differs between endometritis disease classifications in endometrial samples.

Inflammasome receptor mRNA expression levels were quantified in endometrial samples collected at 7 and 21 DPP. Following collection samples were stored at -80°C until total RNA extraction was performed using the TRIzol method. First strand cDNA was reverse transcribed and qPCR for the inflammasome receptors (A.), NLRP3, (B.), IFI16, (C.), NLRC4 and (D.), NLRP1 was performed. Expression levels are given relative to the reference gene H3F3A. Cows were retrospectively classified as being healthy or having endometritis based on cytology scoring of infiltrating neutrophils. Columns indicate mean mRNA expression relative to the reference gene H3F3A, error bars indicate standard error of the mean (7 DPP: healthy n=5, cytological endometritis n=5, cytological endometritis with PVD n=3; 21 DPP: healthy n=5, cytological endometritis n=3, cytological endometritis with PVD n=7). Statistical analysis was performed using a Mann Whitney test between healthy and individual endometritic groups at each time point.

Given the strong expression of IL-1β protein we observed within the endometritic endometrial samples, we next wanted to localize this IL-1β production within the
postpartum endometrium using immunohistochemistry. A previous study by our group had collected endometrial biopsies 7 and 21 DPP from both healthy and endometritic cows. Cows were retrospectively classified as either healthy or as having cytological endometritis based on immune cell infiltration at 21 DPP. The level of inflammation present was assessed based on previously published criteria which examined the extent of inflammatory cell infiltration in both the endometrial epithelium and underlying stromal layers and gave a score from 0-3 based on the intensity of immune cell infiltrate (Chapwanya, Meade et al. 2010, Meira, Henriques et al. 2012). The scores from both the epithelial layer and stromal layer were then combined, with a score ≤ 3 defining healthy cows, while a score ≥ 4 defined cytological endometritis. The results of this scoring are detailed in Table 4.2 and visualised in Figure 4.5.

Table 4.2 Histological scoring of inflammation levels in endometrial biopsies collected at 7 and 21 DPP.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Epithelium</th>
<th>Stroma</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</table>

In the original study the inflammation scoring was performed using haemotoxylin and eosin (H&E) staining to identify polymorphonuclear cells (PMNs). We validated this by immunohistochemical staining for CD45, a marker of haematopoietic immune cells (Figure 4.5). The results of the CD45 staining indicate a clear visual difference in the level of immune cells present between healthy and endometritic cows (Figure 4.5).
Figure 4.5. Example of immune cell localisation in both healthy and endometritic cows.

Endometrial biopsies were stained with haematoxylin and eosin (A. & C.) or stained for CD45 expression by IHC (B. & D.). Endometrial biopsies were collected at 21 DPP and retrospectively classified as either healthy (A.-B.) or endometritic (C.-D.) based on immune cell scoring. Biopsies were formalin fixed and paraffin embedded before being sectioned at 5 μm thickness. Sections were either stained with haematoxylin and eosin or IHC for CD45 was performed. For IHC analysis, Sections were de-paraffinised and rehydrated before antigen retrieval was performed. Following blocking steps, sections were stained using a bovine specific CD45 antibody and positive staining visualised using DAB substrate. Nuclei were counterstained with haematoxylin. Tissue sections were visualised using the Aperio ScanScope Imager under 20x magnification. Scale bar indicates 100 μm.

Localizing CD45+ immune cells within the endometrium, we found that CD45+ immune cell populations tended to localize close to the uterine lumen (Figure 4.6, immune cell margin indicated by black arrows). As seen in Figure 4.6, immune cell populations within healthy cows are reduced by 21 DPP, with distinct clusters remaining close to the uterine lumen. In endometritic cows, large numbers of immune cells are still clearly visible at 21 DPP, dispersed among the upper-functional stroma layer of the endometrium (Figure 4.6 B & D).
Figure 4.6. CD45+ immune cells are located in close proximity to the uterine lumen in the postpartum endometrium.

Endometrial biopsies were stained by IHC for CD45 expression by IHC. Endometrial biopsies were collected at 7 (A.-B.) and 21 DPP (C.-D.) and retrospectively classified as either healthy (A. & C.) or endometritis (B. & D.) based on immune cell scoring at 21 DPP. Biopsies were formalin fixed and paraffin embedded before being sectioned at 5 μm thickness. Sections were subsequently de-paraffinised and rehydrated before antigen retrieval was performed. Following blocking steps, sections were stained using a bovine specific CD45 antibody and positive staining visualised using DAB substrate. Nuclei were counterstained with haematoxylin. Tissue sections were visualised using the Aperio ScanScope Imager under 20x magnification. Scale bar indicates 100 μm. The immune cell margin is indicated by the black arrows.

Together with a qualified veterinarian pathologist, we examined CD45 stained sections in order to identify the immune cell populations in our postpartum endometrial biopsies. Neutrophils, macrophages, NK cells and lymphocytes were all identified (Figure 4.7). Neutrophils were localised to blood vessels or close to the luminal epithelium (Figure 4.7 A.-B.). Some neutrophils could be observed squeezing between the luminal epithelial cells making their way to the uterine lumen. Macrophages localised close to the glandular epithelium (Figure 4.7 C.-D.). Interestingly, macrophages in all endometrial sections, from both healthy and endometritic cows, did not stain positive for CD45. NK cells
(Figure 4.7 E.-F.) and lymphocytes (Figure 4.7 G.-H.) were localised throughout the upper stromal layer.
Figure 4.7. Identification of immune cell populations present in the postpartum endometrium.

Endometrial biopsies were stained by IHC for CD45 expression by IHC. Immune cell populations were identified based on morphology. (A.-B.) neutrophils, (C.-D.) macrophages, (E.-F.) NK cells and (G.-H.) lymphocytes. Endometrial biopsies were collected at 7 and 21 DPP and retrospectively classified as either healthy or endometritic based on immune cell scoring at 21 DPP. Biopsies were formalin fixed and paraffin embedded before being sectioned at 5 μm thickness. Sections were subsequently de-paraffinised and rehydrated before antigen retrieval was performed. Following blocking steps, sections were stained using a bovine specific CD45 antibody and positive staining visualised using DAB substrate. Nuclei were counterstained with haematoxylin. Tissue sections were visualised using the Aperio ScanScope Imager under 20x magnification. Scale bar indicates 100 μm. The immune cell margin is indicated by the black arrows.

We examined the expression of IL-1β and its co-localization with CD45+ immune cells within healthy endometrium postpartum. Within the majority of healthy and endometritic samples, the luminal epithelium is disrupted or in some cases completely absent at 7 DPP. At 7 DPP we observed high numbers of CD45+ immune cells localized in close proximity to the uterine lumen (Figure 4.8 C.). IL-1β expression is localized throughout the endometrial section, not just limited to immune cell populations (Figure 4.8 A. & C.). This indicates a potential role for the epithelium and stromal fibroblasts in mediating IL-1β production. At 21 DPP there is a clear reduction in the level of immune cell infiltrate (Figure 4.8 D.). IL-1β expression begins to resolve to the luminal and glandular epithelium (Figure 4.8 B.). Within the majority of healthy samples, the luminal epithelium was restored by 21 DPP.
Figure 4.8. IL-1β expression is localized to epithelial cells and stromal fibroblasts in biopsies from healthy cows postpartum.

Endometrial biopsies were stained for IL-1β (A. & B.) or CD45 (C. & D.) expression by IHC. Endometrial biopsies were collected at 7 and 21 DPP and retrospectively classified as either healthy (A. & C.) or endometritic (B. & D.) based on immune cell scoring at 21 DPP. Biopsies were formalin fixed and paraffin embedded before being sectioned at 5 μm thickness. Sections were subsequently de-paraffinised and rehydrated before antigen retrieval was performed. Following blocking steps, sections were stained using a bovine specific IL-1β or CD45 antibody and positive staining visualised using DAB substrate. Nuclei were counterstained with haematoxylin. Tissue sections were visualised using the Aperio ScanScope Imager under 20x magnification. Scale bar indicates 100 μm.

The resolution of IL-1β expression to the luminal and glandular epithelium in healthy cows is particularly striking in Figure 4.9 A.-B. Immune cell infiltration has resolved to a number of areas directly adjacent to the uterine lumen (Figure 4.9 C.). Examining the endometrial glands in more detail in Figure 4.9 D. reveals an absence of CD45+ immune cells within this region, confirming IL-1β expression is solely mediated by the glandular epithelial cells.
Endometrial biopsies were stained for IL-1β (A.-B.) or CD45 (C.-D.) expression by IHC. Endometrial biopsies were collected at 7 and 21 DPP and retrospectively classified as either healthy (A. & C.) or endometritic (B. & D.) based on immune cell scoring at 21 DPP. Biopsies were formalin fixed and paraffin embedded before being sectioned at 5 μm thickness. Sections were subsequently de-paraffinised and rehydrated before antigen retrieval was performed. Following blocking steps, sections were stained using a bovine specific IL-1β or CD45 antibody and positive staining visualised using DAB substrate. Nuclei were counterstained with haematoxylin. Tissue sections were visualised using the Aperio ScanScope Imager under 20x magnification. Scale bar indicates 100 μm.

An intact epithelium appears to mediate a protective effect within the endometrium of healthy cows, attenuating the expression of IL-1β (Figure 4.10). Examining sections from a biopsy taken from a healthy cow at 7 DPP, we can observe that directly beneath the luminal epithelium there is a region of stromal fibroblasts and immune cells in which there is a complete absence of IL-1β expression, indicated by the black arrows in Figure 4.10 B. The presence of an intact luminal epithelium is protecting the underlying stroma layer from interaction with any IL-1β priming PAMPs or epithelium is releasing factors aimed at regulating IL-1β expression. IL-1β production within the luminal epithelium appears directed towards the apical membrane (which corresponds to the uterine lumen).
Figure 4.10. Stromal fibroblasts underlying an intact epithelium demonstrate restricted IL-1β expression.

Endometrial biopsies from healthy cows at 7 DPP were stained for IL-1β expression by IHC. Biopsies were retrospectively classified as healthy based on immune cell scoring at 21 DPP. Biopsies were formalin fixed and paraffin embedded before being sectioned at 5 μm thickness. Sections were subsequently de-paraffinised and rehydrated before antigen retrieval was performed. Following blocking steps, sections were stained using a bovine specific IL-1β or CD45 antibody and positive staining visualised using DAB substrate. Nuclei were counterstained with haematoxylin. Tissue sections were visualised using the Aperio ScanScope Imager under 20x magnification. Scale bar indicates 100 μm. Black arrows indicate margin of cell with low IL-1β expression directly beneath the intact luminal epithelium.

Having defined the localisation of IL-1β and CD45 expression within healthy cows, we next examined the localisation of both IL-1β and CD45 within endometritic uterine biopsies. At 7 DPP partial or complete loss of the luminal epithelium was evident in all biopsies from endometritic cows. The loss of luminal epithelium is particularly evident in Figure 4.11. Additionally, the majority of biopsies collected at 7 DPP exhibited a large amount of fibrotic or disrupted tissue (as is evident in Figure 4.11 A. & C.). IL-1β expression appears less intense 7 DPP in cows with CE compared to healthy cows at the same time point (Figure 4.11 A.-B.). CD45+ immune cell expression was again localised in close proximity to the uterine lumen (Figure 4.11 C.). At 21 DPP a significant level of disruption/loss of luminal epithelium still exists. A large immune cell infiltrate remains
in the upper functional layer of the endometrium. Expression of IL-1β remains widespread throughout the tissue (Figure 4.11 B.) and does not appear to resolve to the epithelium as is observed within the healthy cows.

Figure 4.11 IL-1β expression is elevated 21 DPP in epithelial cells and stromal fibroblasts of cows with cytological endometritis.

Endometrial biopsies were stained for IL-1β (A.-B.) or CD45 (C.-D.) expression by IHC. Endometrial biopsies were collected at 7 and 21 DPP and retrospectively classified as either healthy (A. & C.) or endometric (B. & D.) based on immune cell scoring at 21 DPP. Biopsies were formalin fixed and paraffin embedded before being sectioned at 5 μm thickness. Sections were subsequently de-paraffinised and rehydrated before antigen retrieval was performed. Following blocking steps, sections were stained using a bovine specific IL-1β or CD45 antibody and positive staining visualised using DAB substrate. Nuclei were counterstained with haematoxylin. Tissue sections were visualised using the Aperio ScanScope Imager under 20x magnification. Scale bar indicates 100 μm.

The localisation of IL-1β expression to the epithelium at 21 DPP is further evident in Figure 4.12 B. At 7 DPP IL-1β expression is widespread throughout the tissue. Similar to what we observed in Figure 4.11, a level of fibrotic or disrupted tissue is again evident at 7 DPP (Figure 4.12 A. & C.). At 21 DPP disruption to luminal epithelial barrier still
visible (Figure 4.12 B. & D.). IL-1β expression remains widespread throughout the stromal layer at 21 DPP (Figure 4.12 B.). Similarly, there is no reduction in the levels of immune cells present (Figure 4.12 D.).

Figure 4.12. IL-1β expression is localized to the epithelium and stromal fibroblasts at 21 DPP in endometritic cows.

Endometrial biopsies were stained for IL-1β (A.-B.) or CD45 (C.-D.) expression by IHC. Endometrial biopsies were collected at 7 and 21 DPP and retrospectively classified as either healthy (A. & C.) or endometritic (B. & D.) based on immune cell scoring at 21 DPP. Biopsies were formalin fixed and paraffin embedded before being sectioned at 5 μm thickness. Sections were subsequently de-paraffinised and rehydrated before antigen retrieval was performed. Following blocking steps, sections were stained using a bovine specific IL-1β or CD45 antibody and positive staining visualised using DAB substrate. Nuclei were counterstained with haematoxylin. Tissue sections were visualised using the Aperio ScanScope Imager under 20x magnification. Scale bar indicates 100 μm.
4.3.2 IL-1β is produced locally by endometrial epithelial cells and stromal fibroblasts

Having observed high levels of IL-1β expression within luminal and glandular epithelial cells and underlying stromal fibroblasts by IHC staining of endometrial biopsies, we next wanted to examine the constitutive expression and subsequent induction of IL-1β along with other IL-1 family members, namely IL-1α, IL-18, IL-1RN and IL-33 by these cells. We examined cytokine induction at the mRNA level by qPCR and at the protein level using a bovine specific ELISA for IL-β. Constitutive expression was examined in untreated cells by qPCR. Cytokine induction was examined in response to a time course stimulation with inflammasome activating signals, LPS and nigericin. Cells were stimulated with 2.0 µg/ml LPS for 3, 6, 12 and 24 hours followed by stimulation with 10 µM nigericin for 1 hour to activate the inflammasome. Concentrations of LPS and nigericin had previously been optimised following testing with a range of concentrations (0.1-10 µg/ml LPS and 1-20 µM nigericin).

Basal expression of IL1B in epithelial cells (0.4×10^{-3} relative expression units to H3F3A) was comparable to levels seen in stromal cells (0.4×10^{-3}) but significantly lower than what is observed in PBMCs (122×10^{-3}; adjusted p-value <0.0001) (Figure 4.13 A.). IL1A mRNA was expressed across untreated PBMC samples (4.0×10^{-2}), epithelial cells (1.0×10^{-2}) and stromal fibroblasts (1.0×10^{-2}), where values were comparable (Figure 4.13 B.). Constitutive expression of IL18 mRNA was significantly higher in epithelial cell (4.0×10^{-1}; adjusted p-value=0.0013) or stromal fibroblast (9.0×10^{-1}; adjusted p-value=0.0002) compared to PBMC mRNA baseline expression (0.06×10^{-1}) (Figure 4.13 C.). Baseline levels of IL1RN mRNA were comparable across PBMC (2.0×10^{-3}), stromal fibroblast samples (2.0×10^{-3}) and epithelial cells (Figure 4.13 D.). IL33 mRNA was undetectable in PBMC and epithelial cell samples, but was detectable across all stromal fibroblast samples (1.0×10^{-2}) (Figure 4.13 E.).
Figure 4.13: Endometrial cell populations display different baseline expression levels of IL-1 family members.

(A.) IL1B, (B.) IL1A, (C.) IL18, (D.) IL1RN and (E.) IL33 mRNA baseline expression relative to H3F3A across unstimulated samples. Cells were harvested in TRizol, RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene H3F3A. Columns indicate mean expression relative to H3F3A, error bars indicate standard error of the mean (n=5). Statistical analysis was performed using a Kruskal Wallis test with Dunns multiple comparison test. *=p≤0.05; **=p≤0.01; ***=p≤0.001; ****=p≤0.0001.
Having examined their constitutive expression in unstimulated control samples, we next examined the subsequent induction of *IL1A*, *IL18*, *IL1RN* and *IL33* at the mRNA level in response to a time course stimulation with inflammasome activating signals, LPS and nigericin as detailed previously.

*IL1A* mRNA induction in PBMCs peaked significantly at 12 hours post-stimulation with a MFC of 21.4 (p= 0.0286) (Figure 4.14 A.). *IL1A* mRNA was not strongly induced in epithelial cells in response to LPS and nigericin with *IL1A* mRNA induction peaking at 24 hours post stimulation with a MFC of 2.6 (Figure 4.14 B.). *IL1A* induction by stromal fibroblasts peaked at 6 hours post-stimulation with a MFC of 99.5 before reducing to a MFC of 8.4 at 24 hours post-stimulation (Figure 4.14 C.). As *IL18* is constitutively expressed at the mRNA level, examining its mRNA induction is of limited value in elucidating its key producers in the endometrium. *IL18* was not induced at the mRNA level in response to LPS and nigericin across PBMC, epithelial cell or stromal fibroblast samples (Figure 4.14 D.-F.). *IL1RN* saw the highest level of induction with a MFC of 49.0 at 6 hours post-stimulation in PBMCs (Figure 4.14 G.). Induction of *IL1RN* in epithelial cells was significantly higher at 24 hours post-stimulation with a MFC of 1.8 (p=0.0184) (Figure 4.14 H.). *IL1RN* is not induced in stromal fibroblasts response to LPS and nigericin stimulation (Figure 4.14 I.). No expression or induction of *IL33* was observed in PBMC or epithelial cell samples (Figure 4.14 J.-K.). *IL33* was significantly induced in stromal fibroblasts at 3 hours post-stimulation with a MFC of 28.5 (p=0.0286) before induction levels dropped to a MFC of 0.8 at 6 hours post stimulation (Figure 4.14 L.).
Figure 4.14. IL-1 family members display differential induction patterns in endometrial cell populations.

(A.-C.). *IL1A*, (D.-F.) *IL18*, (G.-I.) *IL1RN* and (J.-L.) *IL33* mRNA fold change in PBMCs, epithelial cells and stromal fibroblasts following stimulation with a combination of LPS (2 µg/ml) and nigericin (10 µM). Following stimulation cells were harvested in TRIzol for gene expression analysis. RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene *H3F3A*. Columns indicate mean fold change relative to unstimulated control, error bars indicate standard error of the mean (*n*=5). Statistical analysis was performed using a Mann Whitney test, comparing untreated control to a timepoint stimulation. *=p≤0.05.
We next examined the induction of IL-1β in response to the inflammasome priming signal LPS alone (2 µg/ml), the inflammasome activating signal nigericin alone (10 µM), or a combination of the two stimuli over a time course of 24 hours. IL-1β induction demonstrated distinct expression patterns in each of the cell types examined. Investigating IL1B induction at the mRNA level, we found that stimulation with LPS alone or in combination with the inflammasome activating ligand nigericin was sufficient to induce IL1B expression across all three cell populations. Stimulation with nigericin alone had no effect on IL1B expression across the three cell populations examined. IL1B induction occurred at the later timepoints of 12 and 24 hours post stimulation in PBMCs. Induction of IL1B peaked significantly at 12 hours post-stimulation with a MFC of 30.1 in LPS stimulated cells and a MFC of 30.3 in cells stimulated with LPS and nigericin (p=0.0057) (Figure 4.15 A.). Epithelial cell induction of IL1B mRNA was stronger at earlier time points, with significant induction of IL1B occurring at 6 hours post stimulation (MFC of 71.0 in LPS treated and a MFC of 62.6 in LPS and nigericin stimulated cells, p=0.0291). However similar to PBMCs, peak induction occurred at 12 hours post-stimulation with a MFC of 77.9 in LPS and nigericin stimulated cells (p=0.0291) (Figure 4.15 B.). IL1B is strongly induced in stromal fibroblasts with a statistically significant (p=0.0025) peak MFC of 80.9 following LPS stimulation and a MFC of 85.8 following LPS and nigericin stimulation occurring at 6 hours post-stimulation (Figure 4.15 C.).

Examining IL-1β protein expression by ELISA revealed that stimulation with both inflammasome signal, LPS and nigericin, is required for IL-1β protein secretion across all three cell populations. Examining the expression of IL-1β protein release from PBMCs we observe peak levels of protein expression at 12 hours post stimulation (1089.9 pg/ml versus 12.8 pg/ml in unstimulated control samples, p=0.0061). Levels of protein expression remain significant at 24 hours post stimulation (355.9 pg/ml in samples treated with LPS and nigericin, p=0.0016) (Figure 4.15 D.). IL-1β production in epithelial cells peaked significantly at the earlier time points of 3 (1309.8 pg/ml; p=0.0381) and 6 (2200.6 pg/ml; p=0.0095) hours post stimulation (Figure 4.15 E.). This level of protein expression is comparable to what is observed in PBMCs at later time points treated with similar concentrations of LPS and nigericin. Stromal fibroblasts demonstrated the highest expression of IL-1β, with this production sustained over the 24 hour time period (Figure 4.14 F.). Peak IL-1β production in stromal fibroblasts occurred at 6 hours post stimulation.
(4604.9 pg/ml; p=0.0444), although all time points exhibit significant levels of IL-1β production compared to the unstimulated control. Stromal cells display a higher expression of IL-1β protein when compared to both epithelial (where peak expression was 2200.6 pg/ml) and PBMCs (where peak expression was 1089.9 pg/ml).
Figure 4.15: IL-1β production is induced in endometrial cells in response to LPS and the inflammasome activating signal nigericin. (A., B., C.). *IL1B* mRNA fold change and (D., E., F.). IL-1β protein expression in PBMCs, epithelial cells and stromal fibroblasts following stimulation with LPS (2 µg/ml) or nigericin (10 µM) or a combination of LPS and nigericin. Following stimulation cells were harvested in TRIzol for gene expression analysis and cell supernatants were harvested for analysis of protein production. RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene *H3F3A*. IL-1β protein levels were examined using a bovine specific IL-1β ELISA. For qPCR results columns indicate mean fold change relative to unstimulated control, for ELISA results columns indicate mean cytokine expression per ml of media, error bars indicate standard error of the mean (n=5). Statistical analysis was performed using a Mann Whitney test, comparing the untreated control to a timepoint stimulation. *=p≤0.05; **=p≤0.01.
While it is clear that endometrial epithelial and stromal fibroblasts play a role in mediating IL-1β production in the endometrium, examining the mRNA induction and subsequent protein production of IL-1β in epithelial cells using the current 2D stimulation models may not give a true reflection the ability of these cells to produce IL-1β, given the fact that epithelial cells maintain a 3D structure in-vivo. The relatively high basal level of protein expression observed in the control samples by ELISA (185.1 pg/ml compared to 12.8 pg/ml in PBMC control samples) indicate that these cells are primed to respond when given the appropriate signal. Further investigation using a more physiologically relevant culture system (i.e. a model of polarized epithelial cells) is required to fully elucidate the role endometrial epithelial cells play in IL-1β production.
4.3.3 IL-1β is differentially induced in polarized epithelial cells

One limitation of our previous study examining IL-1β induction in our primary epithelial cell stimulations is the fact that in-vivo, endometrial epithelial cells possess a 3D structure maintained by the tight junctions between the epithelial cells. This allows them to polarize and respond differently to antigens presented at either the apical or basolateral membrane. Our previous study examining epithelial cell responses only accounted for antigens presented to the apical membrane. To address this issue, we established a model of polarized epithelial cells grown on transwell inserts which allow us to mimic their 3D in-vivo properties and stimulate these cells from either the apical or basolateral membrane.

Having previously confirmed that our polarized epithelial cultures differentially respond to stimulation with PAMPs at either the apical or basolateral membrane, we wanted to investigate the effect of differential stimuli on IL-1β induction in our polarized epithelial cells. Epithelial cells were grown to confluence on transwell inserts coated with a thin layer of matrigel and allowed to polarize over the course of 2 weeks. The polarized epithelial cells were then stimulated either apically or basolaterally with 2.0 µg/ml LPS for 3, 6, 12 and 24 hours followed by stimulation with 10 µM nigericin to activate the inflammasome and allow for IL-1β protein secretion.

Polarized epithelial cells treated apically with LPS and nigericin exhibit weak induction of IL1B with a peak MFC of 4.3 occurring at 12 hours post-stimulation (Figure 4.16). However basolateral induction of the polarized epithelial cells results in a much stronger induction of IL1B mRNA. IL1B mRNA induction was significantly higher at 24 hours post-stimulation in the basolaterally stimulated cells with a MFC of 179.1 (p=0.0019) compared to the apically stimulated cells which had a MFC of 2.0. Induction of IL1B was also higher at both 6 hours (basolateral MFC was 11.4 versus 2.4 in apically treated cells) and 12 hours post-stimulation (basolateral MFC was 11.1 versus 4.4 in apically treated cells).
**Figure 4.16: IL1B mRNA is induced following basolateral stimulation in polarized epithelial cells.**

IL1B mRNA fold change following stimulation. Epithelial cells were grown to confluency on transwell inserts coated with matrigel and allowed to polarize before stimulation with LPS and nigericin in either the apical or basolateral compartment. Following stimulation, the cells were harvested in TRIzol. Total RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene H3F3A. Columns indicate mean fold change relative to unstimulated control, error bars indicate standard deviation (n=5). Statistical analysis was performed using a Mann Whitney test comparing untreated control to a timepoint stimulation. **=p≤0.01.

Having observed significantly higher induction of IL1B as a result of basolateral stimulation, we next investigated the induction of both the TLR4 receptor and the NLRP3 receptor (**Figure 4.17**). There was no significant difference between the induction of TLR-4 as a result of apical or basolateral stimulation (**Figure 4.17 A.**). Peak TLR4 induction occurs at 12 hours post-stimulation with the apically stimulated levels (MFC of 3.0) comparable to the basolaterally stimulated levels (MFC of 3.2). Similarly, induction of NLRP3 mRNA was not significantly different between the stimulations across all time points (**Figure 4.17 B.**). Peak NLRP3 induction occurred at 3 hours post-stimulation with no observable difference between the apically stimulated cells (MFC of 7.8) compared to the basolaterally stimulated cells (MFC of 6.4).
A. **TLR4** mRNA Expression in Polarized Epithelial Cells

![Graph showing TLR4 mRNA expression](image)

**Figure 4.17**: TLR4 and NLRP3 receptor mRNA are not differentially induced in polarized epithelial cells.

(A.). **TLR4** and (B.). **NLRP3** mRNA fold change following stimulation. Epithelial cells were grown to confluence on transwell inserts coated with matrigel and allowed to polarize before stimulation with LPS in either the apical or basolateral compartment. Following stimulation, cells were harvested in TRIzol. RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene H3F3A. Columns indicate mean fold change relative to unstimulated control, error bars indicate standard error of the mean (n=5). Statistical analysis was performed using a Kruskal Wallis test with Dunns multiple comparison test.

Having demonstrated that **IL1B** mRNA induction was differentially induced in response to basolateral stimulation in our polarized epithelial cell model, we next wanted to examine whether this was reflected at the protein level and if IL-1β was preferentially secreted from either the apical or basolateral membrane. Following our time course stimulation with 2 µg/ml LPS and 10 µM nigericin, supernatants were harvested and IL-1β levels were quantified using a bovine IL-1β ELISA kit.

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Examining whether apical or basolateral stimulation induced higher levels of IL-1β protein expression, we see elevated levels of IL-1β accumulating in the apical compartment of epithelial cells stimulated basolaterally (1194.81 pg/ml) compared to cells stimulated apically (529.0 pg/ml) 6 hours post-stimulation (Figure 4.18). This is reflective of what we observed examining IL1B mRNA induction and indicates that IL-1β is preferentially expressed following basolateral stimulation in endometrial epithelial cells.

We next examined the preferential route of secretion of the IL-1β cytokine. Measuring IL-1β levels in both the apical and basolateral compartment following stimulation, we see higher levels of IL-1β accumulating in the apical compartment across all time-points following basolateral stimulation (Figure 4.18 A.). Peak IL-1β expression occurred at 6 hours post-stimulation with 1194.81 pg/ml IL-1β accumulating in the apical compartment compared to 305.985 pg/ml accumulating in the basolateral compartment. Examining IL-1β expression following apical stimulation we see a similar trend with higher IL-1β levels accumulating in the apical compartment across all time-points (Figure 4.18 B.). Peak IL-1β expression occurred at 6 hours post-stimulation with 529.0 pg/ml IL-1β accumulating in the apical compartment compared to 384.8 pg/ml in the basolateral compartment.

These results indicate that, while IL-1β is preferentially induced through stimulation at the basolateral membrane, secretion of this cytokine is from the apical membrane, directing the cytokine into the uterine lumen. As IL-1β has classically been considered to lack a signalling peptide, the mechanism directing its secretion to the apical membrane remains unknown.
IL-1β expression is directionally secreted towards the apical compartment by polarized epithelial cells.

IL-1β protein expression and directional secretion following stimulation. Epithelial cells were grown to confluency on transwell inserts coated with matrigel and allowed to polarize before stimulation with LPS and nigericin in either the apical or basolateral compartment. Following stimulation, the supernatants were collected and the levels of IL-1β were quantified using a commercially available bovine specific IL-1β ELISA kit. Columns indicate mean cytokine expression per ml of media, error bars indicate standard error of the mean (n=5). Statistical analysis was performed using a Mann Whitney test, comparing untreated control to a timepoint stimulation.
4.3.4 A novel variant of IL-1β is produced by endometrial epithelial cells

Examining the protein expression of pro-IL-1β in epithelial cells by western blotting, we identified a number of anomalies. Similar to what has previously been reported in bovine PBMCs (Harte, Gorman et al. 2017), unstimulated endometrial epithelial cells contain pre-formed IL-1β (Figure 4.19) which our ELISA data shows, can be released from the cell in response to nigericin alone (Figure 4.15). In addition, we have consistently observed a band at a higher than expected molecular weight, with a pro-IL-1β band appearing at 43-55 kDa rather than at the expected 35 kDa which is observed in PBMC lysates run on the same gel (Figure 4.19). A similar issue was observed when examining IL-1β expression in endometrial samples collected by cytobrush (Figure 4.3), where bands corresponding to pro- and active-IL-1β were detected in addition to extra bands corresponding to a high molecular weight isoform of both pro- and active IL-1β. The presence of this variant was validated through the use of two different, commercially available, bovine specific IL-1β antibodies, purchased from two separate companies.
Figure 4.19. A high molecular weight isoform of IL-1β is present in epithelial cell lysates.

Following stimulation, $1 \times 10^6$ endometrial epithelial cells were harvested in RIPA lysis buffer. Total protein levels were measured using a BCA assay and 20 µg of protein was loaded on an SDS-PAGE gel. Gel electrophoresis was performed and the resulting gel was electrophoretically transferred onto a PVDF membrane before incubation in blocking buffer (5% milk powder in PBS-T) for 1 hour followed by incubation overnight at 4°C with an antibody directed against either IL-1β (cytokine of interest) or β-actin (loading control). The membrane was washed three times in PBS-T before incubation for 1 hour at room temperature with the appropriate secondary antibody conjugated to HRP. The membrane was then washed a further three times before visualisation using the Biorad ChemiDoc MP system and HRP substrate solution (ECL).

Examining pro-IL-1β expression in stromal fibroblasts, we found IL-1β to be located at the correct, predicted molecular weight of 35 kDa (Figure 4.20). IL-1β was detected in PBMC lysates at the same size. Similar to what we observed in epithelial cell lysates and what has previously been reported in PBMCs, unstimulated stromal fibroblasts contain pre-formed IL-1β, of which small amounts are released when cells are stimulated with nigericin alone.
Figure 4.20. IL-1β is located at the correct molecular weight in stromal fibroblast lysates.

Following stimulation, $1 \times 10^6$ endometrial stromal fibroblasts were harvested in RIPA lysis buffer. Total protein levels were measured using a BCA assay and 20 µg of protein was loaded on an SDS-PAGE gel. Gel electrophoresis was performed and the resulting gel was electrophoretically transferred onto a PVDF membrane before incubation in blocking buffer (5% milk powder in PBS-T) for 1 hour followed by incubation overnight at 4°C with an antibody directed against either IL-1β (cytokine of interest) or β-actin (loading control). The membrane was washed three times in PBS-T before incubation for 1 hour at room temperature with the appropriate secondary antibody conjugated to HRP. The membrane was then washed a further three times before visualisation using the Biorad ChemiDoc MP system and HRP substrate solution (ECL).

Given the direction secretion of IL-1β towards the apical membrane observed in our polarized epithelial cell cultures, we hypothesized that pro-IL-1β may be tagged with a signal peptide directing it to the apical cell membrane for release. However, this is in conflict with previously published observations on human and mouse IL-1β which state that IL-1β lacks a signal peptide to direct it to the apical membrane. We identified a number of possible mechanisms, either at the transcriptional or post-translational stage, by which IL-1β might be modified to the high molecular weight variant identified in epithelial cells (detailed in Table 4.3).
Table 4.3. Summary of possible mechanisms for modification of IL-1β in epithelial cells.

<table>
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<tr>
<th>Stage of Modification</th>
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<td>Post-translational</td>
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<td>• Forming a dimer.</td>
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<tr>
<td>Other</td>
<td>• Cross reacting with another IL-1 family member.</td>
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We began by examining available genomic data for bovine *IL1B*. *IL1B* is located on chromosome 11 in the bovine and is composed of 7 exons and 6 introns. Expressed sequence tag (EST) analysis was used to bioinformatically predict splice variants present in the bovine. ESTs are short (200-800 nucleotide bases in length), unedited sequence reads generated from cDNA libraries. ESTs have played an instrumental role in gene transcript discovery in the past. We identified a number of *IL1B* splice variants existing in the bovine (Figure 4.21), although none of these splice variants none of these bioinformatically predicted splice variants correspond to a protein of the size observed in our endometrial epithelial cell lysates (predicted protein sizes: splice variants one: 22.7 kDa, splice variant two: 27.1 kDa, splice variant three: 18.7 kDa, splice variant four: 26.1 kDa, splice variant five: 20.4 kDa, splice variant six 20.3 kDa). However, these represent only the reported splice variants. Other splice variants may exist but due to the limited work on bovine *IL1B* they may not have been reported on yet.
Figure 4.21. A number of *IL1B* splice variants are predicted to exist within the bovine.

Bovine *IL1B* is composed of 7 exons and 6 introns. Expressed sequence tag (EST) analysis was used to identify alternative splices of the bovine *IL1B* gene. Six ESTs relating to *IL1B* were identified using the UCSC genome browser. Black squares indicate exons while thin lines indicate introns. Scale bar indicates 100 bases.

A PCR assay was established to validate expression of predicted splice variants and probe for novel unannotated splice variants in epithelial cells. A primer set spanning exons 1-7 was designed and optimised for optimal primer concentration and annealing temperature. The primer spanning region is indicated by the blue arrows (Figure 4.22 A.). Gels were run with either a 2,000 bp DNA ladder (spanning 0.1-2.0 kb) (Figure 4.22 B.) or 10,000 bp DNA ladder (spanning 0.1-10.0 kb) (Figure 4.22 C.). A number of splice variants were identified across the three cell populations, with epithelial cells possessing 7 splice variants compared to 5 in stromal fibroblasts and 2 in PBMCs, although the full length, un-spliced transcript demonstrated the strongest level of expression in all three cell populations. None of the identified splice variants were larger in size (and thus corresponding to our higher molecular weight protein isoform) than our full length *IL1B*
product, indicating that our high molecular weight variant is not generated at the transcriptional level.

Figure 4.22. Multiple IL1B splice variants are expressed in endometrial epithelial cells and stromal fibroblasts.

Expression of full length IL1B was determined using primers to span the entire IL1B transcript. Primer pairs used are indicated by the blue arrows in (A.). Following LPS stimulation for 6 hours, cells were harvested in TRIzol for gene expression analysis. RNA was extracted and first strand cDNA reverse transcribed. PCR for full length IL1B was carried out and the resulting amplifications were visualised using 2% agarose gel electrophoresis ran with either a 2.0 kb DNA ladder (B.) or a 10.0 kb DNA ladder (C.). Splice variants are indicated by the white arrows.

With no evidence for transcriptional modification of IL-1β in epithelial cells, we began to examine potential mechanisms of post-translational cytokine modification. Previous
reports had identified the process of glycosylation as an important mechanism for the sorting of secreted proteins to the apical or basolateral membrane within epithelial cells (Yamamoto, Awada et al. 2013). We began investigating the glycosylation profile of bovine IL-1β by using publically available bioinformatic tools, NetOGlyc and NetNGlyc, (available at http://www.cbs.dtu.dk/services/NetOGlyc/) to examine predicted glycosylation sites within the IL-1β protein sequence. Bioinformatic analysis identified one possible N-linked glycosylation sites in the bovine IL-1β protein sequence, located as Asp-58 (Figure 4.23). However, this site is located in the pro-form of the cytokine which is thought to be cleaved in order to release the active form of the cytokine from the cell. No predicted sites for O-linked glycosylation were identified in bovine IL-1β.

Figure 4.23. A potential N-linked glycosylation site is located in the bovine IL-1β sequence.

An N-linked glycosylation site was predicted in the bovine IL-1β protein sequence using the bioinformatics tool NetNGlyc. Asn-Xaa-Ser/Thr sequons are shown in blue. Asparagines predicted to be N-glycosylated are shown in red. The graph illustrates the predicted N-glycosylation sites across the protein chain (the x-axis represents protein length from N- to C-terminal). A site of potential N-linked glycosylation is indicated by a vertical line.
Within the human IL-1β sequence, similar to the bovine, only one potential N-linked glycosylation site was predicted (Figure 4.24). However, unlike in the bovine, this predicted site of N-linked glycosylation is located in the active, cleaved part of the cytokine.

![NetNGlyc prediction](image)

**Figure 4.24. A potential N-linked glycosylation site is located in the human IL-1β cytokine.**

An N-linked glycosylation site was predicted in the human IL-1β protein sequence using the bioinformatics tool NetNGlyc. Asn-Xaa-Ser/Thr sequons are shown in blue. Asparagines predicted to be N-glycosylated are shown in red. The graph illustrates the predicted N-glycosylation sites across the protein chain (the x-axis represents protein length from N- to C-terminal). A site of potential N-linked glycosylation is indicated by a vertical line.

In order to investigate the glycosylation status of IL-1β, samples were treated with commercially available protein de-glycosylation mix (New England Biolabs). The protein de-glycosylation mix contains PNGase F, O-glycosidase, Neuraminidase, β1-4
Galactosidase and β-N-acetylglucosaminidase. These enzymes remove all carbohydrates but don’t determine the nature of the glycosylation. A positive control protein (fetuin) was also included. Both the positive control protein fetuin and epithelial cell lysate were incubated with the de-glycosylation enzyme mix for 16 hours before proteins were visualised by either Coomassie staining (in the case of the fetuin protein as we did not have a fetuin antibody to examine its expression by western blotting) or by western blotting for IL-1β (Figure 4.25). The de-glycosylation mix was successful in removing all glycosylated carbohydrates from the fetuin protein (indicated by the black arrows), reducing it in size from approximately 60 kDa to approximately 40 kDa as visualised on a Coomassie stained gel (Figure 4.25 A.). However, the de-glycosylation enzyme mix was found to have no effect on the high molecular weight variant of IL-1β (band indicated by black arrow), which did not reduce in size (predicted IL-1β size indicated by black arrow) following incubation with the glycosylation stripping enzymes, indicating that glycosylation is not the reason for the larger size of IL-1β in epithelial cells (Figure 4.25 B.).
Figure 4.25. De-glycosylation enzymes have no effect on reducing IL-1β size in endometrial epithelial cells.

Following stimulation, $1 \times 10^6$ endometrial stromal epithelial cells were harvested in RIPA lysis buffer. The control protein fetuin (A.) or epithelial cell lysates (B.) were treated with the de-glycosylation enzyme mix for 16 hours at 37°C. 20 µg of protein was loaded on an SDS-PAGE gel. Gel electrophoresis was performed and the resulting gel was either stained with Coomassie dye (for fetuin visualisation) or electrophoretically transferred onto a PVDF membrane before incubation in blocking buffer (5% milk powder in PBS-T) for 1 hour followed by incubation overnight at 4°C with an antibody directed against IL-1β. The membrane was washed three times in PBS-T before incubation for 1 hour at room temperature with secondary antibody conjugated to HRP. The membrane was then washed a further three times before visualisation using the BioRad ChemiDoc MP system and HRP substrate solution (ECL). Following staining with the Coomassie dye for 1 hour, the gel was de-stained with Coomassie de-stain solution for 2 hours until stain was removed from the background. The gel was subsequently scanned using the Odyssey Infared Imaging System.
Having ruled out glycosylation as a possible mechanism for IL-1β modification in epithelial cells, we next investigated if IL-1β was appearing at a higher molecular weight due to the cytokine binding to a carrier protein or forming a dimer. The presence of a dimer was ruled out due to the size of our variant (55 kDa while a dimer of pro-IL-1β would be approximately 70 kDa). In order to determine if the variant size was due to the binding of a carrier protein, epithelial cell lysates were boiled in reducing conditions (β-mercaptoethanol) for 1 hour at 95°C in order to disrupt disulphide bridges. Boiling the samples for 1 hour at these conditions had no effect on IL-1β size but did appear to damage the protein (as is evidenced in the β-actin band) (Figure 4.26). Extended denaturing conditions involving a 24 hour incubation had no effect on IL-1β size. If the IL-1β variant we have identified is due to the binding of a carrier protein, it appears to be highly resistant to standard methods of dissociation.

![Figure 4.26. Boiling had no effect on reducing the size of IL-1β in epithelial cells.](image)

Following stimulation, 1×10^6 endometrial epithelial cells were harvested in RIPA lysis buffer. Total protein levels were measured using a BCA assay and 20 µg of protein (untreated or boiled for 1 hour in 4X TRIS Glycine SDS β-mercaptoethanol) was loaded on an SDS-PAGE gel. Gel electrophoresis was performed and the resulting gel was electrophoretically transferred onto a PVDF membrane before incubation in blocking buffer (5% milk powder in PBS-T) for 1 hour followed by incubation overnight at 4°C with an antibody directed against either IL-1β (cytokine of interest) or β-actin (loading control). The membrane was washed three times in PBS-T before incubation for 1 hour at room temperature with the appropriate secondary antibody conjugated to HRP. The membrane was then washed a further three times before visualisation using the Biorad ChemiDoc MP system and HRP substrate solution (ECL).
We also investigated the possibility that the IL-1β antibody may be cross reacting with another member of the IL-1 family which shared similar protein homology. The IL-1 family members closest in size were aligned using the BLAST software to give a percentage homology score (detailed in Table 4.4). Bovine IL-1 family members do not display high levels of homology between members (with values ranging from 26-54%) and so this was not considered to be the cause of our high molecular weight IL-1β isoform. Additionally, the other IL-1 family members do not possess the correct size to be identified as our IL-1 variant.

Table 4.4. Protein homology between bovine IL-1 family members.

<table>
<thead>
<tr>
<th>Bovine IL-1 Family Homology</th>
<th>IL-1β</th>
<th>IL-1α</th>
<th>IL-18</th>
<th>IL-33</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>100%</td>
<td>26%</td>
<td>20%</td>
<td>39%</td>
</tr>
<tr>
<td>IL-1α</td>
<td>26%</td>
<td>100%</td>
<td>22%</td>
<td>54%</td>
</tr>
<tr>
<td>IL-18</td>
<td>20%</td>
<td>22%</td>
<td>100%</td>
<td>43%</td>
</tr>
<tr>
<td>IL-33</td>
<td>39%</td>
<td>54%</td>
<td>43%</td>
<td>100%</td>
</tr>
</tbody>
</table>

While it is clear that none of our efforts here were able to elucidate the nature of the IL-1β variant identified in epithelial cells, we aimed to isolate the IL-1β variant and have it analysed by mass spectrometry. Efforts to immunoprecipitate the protein failed and we were unable to separate our IL-1β variant from the rest of the protein in the cell lysates. Despite a significant level of troubleshooting with the immunoprecipitation assay, using different IL-1β bovine antibodies, increasing cell numbers, and antibody incubation times, the antibodies used were unable to isolate our IL-1β variant for mass spectrometry analysis. This could be due in part to the fact that in some cases antibodies cannot detect proteins in their native form (with the immunoprecipitation protocol requiring that the protein be maintained in its native form).

While currently limited by the reagents available for bovine IL-1β research and the limited number of studies characterising the bovine proteome, it is our hope that future studies characterising the bovine proteome will be able to shed light on the nature of our high molecular weight IL-1β variant.
4.3.5 IL-1β negatively affects the re-establishment of the epithelial barrier

Having observed local production of IL-1β by endometrial epithelial cells and stromal fibroblasts and having identified the physiological relevant concentrations of IL-1β in either the healthy or endometritic uterus, we next wanted to investigate the effects of IL-1β was having in the uterus and if this was linked to pathology. Following the process of parturition, the luminal epithelium experiences a significant level of disruption. Re-establishment of the barrier during the processes of tissue involution and repair is crucial to restore the uterus to a state of homeostasis. The effect of IL-1β on epithelial barrier function had previously been investigated in a model of intestinal epithelial cells where it was found to affect barrier function (Al-Sadi and Ma 2007). However, studies with endometrial epithelial cells are lacking. As we observed the strongest level of IL-1β production by stromal fibroblasts, we hypothesised that epithelial cells were more likely to encounter pathological levels of IL-1β at their basolateral membrane and so using our model of polarized epithelial cells we were able to examine the effect on barrier function of basolateral treatment with IL-1β.

We examined the effect of increasing concentrations of IL-1β (5-50 ng/ml) on an established epithelial barrier. Treatment with IL-1β over a time course of 96 hours had no effect on epithelial barrier integrity (Figure 4.27 A.) as measured by the trans-epithelial resistance (TER) or on epithelial barrier permeability as measured by the movement of a FITC labelled dextran molecule across the epithelial barrier (Figure 4.28 B.). Treatment of cell with 20% DMSO completely disrupted the epithelial barrier as is evidenced by the drop in the TER value and an increase in the barrier permeability to FITC-dextran.
Figure 4.27. Increasing concentrations of IL-1β have no effect on an established epithelial barrier.

Epithelial cells were grown to confluency on transwell inserts coated with matrigel and allowed to polarize before treatment with IL-1β (A.). Trans-epithelial resistance was used to examine the permeability of/disruption to the epithelial barrier following treatment with increasing concentrations of IL-1β. (B.). Permeability of the polarized epithelial barrier was quantified by measuring the trans-epithelial flux of a 4 kDa FITC labelled dextran molecule. Briefly, 200 µg of FITC-dextran was added to the apical surface of the monolayer. Following incubation at 37°C for 3 hours, a 200 µl aliquot of media from the basolateral compartment was analysed for FITC fluorescence. Values are expressed as mean percentage of the no cell control, n=3. Error bars not shown for clarity.

Having observed no effect of IL-1β stimulation on an intact epithelial barrier, we next investigated the effect of IL-1β on the re-establishment of the epithelial barrier, similar to what is occurring in the immediate postpartum period during the processes of uterine involution and tissue repair. Epithelial cells were plated on transwell inserts and allowed to grow to confluency and polarise before the addition of the chelating agent EDTA to deplete calcium levels. Depleting calcium levels results in the tight junctions breaking apart. We are then able to add calcium back into the media and observe the tight junctions re-establish in real time in untreated inserts or inserts treated with increasing concentrations of IL-1β. Stimulation with increasing concentrations of IL-1β in either the apical or basolateral compartment impaired barrier recovery compared to the untreated control (Figure 4.28). During apical stimulation, the impairment occurred in a concentration dependent manner, with higher concentrations of IL-1β having a more discernible effect on barrier recovery (Figure 4.28 A.). In contrast, stimulation with higher concentrations basolaterally, while still impairs barrier recovery, had less of an effect than lower concentrations (Figure 4.28 B.).
Figure 4.28. IL-1β negatively affects the re-establishment of the epithelial barrier.

Epithelial cells were grown to confluency on transwell inserts coated with matrigel and allowed to polarize. Polarized epithelial cells were treated with 2 mM EDTA for 5 min to disrupt the tight junctions before being switched to HBSS with normal Ca\textsuperscript{2+} (1.8 mM) and left untreated (control) or incubated with increasing concentrations of IL-1β (5-50 ng/ml) in either the (A.) apical or (B.) basolateral compartment. The re-establishment of the epithelial barrier was measured by monitoring TER over time. Data represents percent recovery over time relative to baseline values, n=3. Error bars not shown for clarity.

The ability of IL-1β to delay the re-establishment of the epithelial barrier demonstrates a clear role for the potent inflammatory cytokine during pathological uterine inflammation postpartum. In order to develop targeted immunotherapies for uterine disease, there is a need for a greater understanding of the events surrounding the regulation and production of this potent inflammatory cytokine in the postpartum uterus.
4.4 Discussion

A role for IL-1 family members in pathology has been widely studied in mouse and human models of inflammatory disease, with IL-1β production in particular implicated in human inflammatory pathologies such as the inherited cryopyrin-associated auto-inflammatory syndrome Muckle Wells syndrome, rheumatoid arthritis as well as a number of metabolic disorders such as type 2 diabetes and gout (Agostini, Martinon et al. 2004, Wen, Ting et al. 2012). Within the bovine, IL-1β has been associated with the immune response of cattle infected with mycobacterium avium subspecies paratuberculosis, the causative agent of Johne’s disease (Casey, Meade et al. 2015). Similarly, a role for IL-1β has been associated with resistance to mycobacterium tuberculosis infection and bovine mastitis (Bourigault, Segueni et al. 2013, Xu, Dong et al. 2018). Production of IL-1β at mucosal sites such as the intestine has been examined by numerous studies in the past, with evidence emerging of a role for the intestinal epithelial cell in mediating IL-1β production (Lei-Leston, Murphy et al. 2017). Recently there has also been an increasing awareness for the role of fibroblasts in mediating inflammatory responses, with fibroblasts implicated in mediating chronic inflammation in rheumatoid arthritis (Mizoguchi and Slowikowski 2018). Limited work has been performed in livestock models but bovine endometrial epithelial cells and stromal fibroblasts have been shown to produce IL-6 and IL-8 in response to inflammatory stimuli (Cronin, Turner et al. 2012, Healy, Cronin et al. 2015). However, while evidence of a role for IL-1β in uterine disease has emerged in recent years, with elevated gene expression of IL-1β reported in endometrial biopsies from cows with endometritis and elevated IL-1β protein levels observed in cervico-vaginal mucus from cows with endometritis at 7 and 21 DPP, the role of IL-1β in mediating the switch to a pathological inflammatory phenotype observed in uterine disease and the cell populations producing IL-1β locally in the uterus remain unknown (Foley, Chapwanya et al. 2015, Adnane, Chapwanya et al. 2017).

Our study is the first to report elevated IL-1β protein levels within the postpartum endometrium, demonstrating a clear difference in expression levels at 7 DPP. Additionally, total protein yields varied between the different disease categories, with visibly higher levels of total protein yields observed in cows with cytological endometritis at 7 DPP (possibly due to an increase in the expression of tissue repair
proteins). Taken together, both total protein yields and IL-1β levels hold promise as potential prognostic biomarkers of endometritis. Examining the expression of the different inflammasome receptors, we observed the strongest expression of NLRP3 within endometrial samples, confirming that IL-1β production within the endometrium is primarily NLRP3 mediated. This had implications for our later work, characterising the regulatory mechanisms surrounding IL-1β cleavage and release from the cell. As IL-1β levels were elevated in both sub-clinical and clinical forms of uterine disease, the discussion here does not discriminate between the two forms if uterine disease but refers to them collectively as ‘endometritis’.

Localising IL-1β expression by immunohistochemistry to the epithelium and stromal fibroblasts demonstrates the predominant local production of IL-1β by the innate cells of the endometrium, as opposed to the invading immune cells. Staining for CD45, a marker of haematopoietic immune cells, demonstrated their localisation in close proximity to the uterine lumen at 7 DPP. Sampling of the uterine lumen by endometrial cytobrushes in another study by our lab has revealed a strong localisation of immune cells, neutrophils in particular, to the uterine lumen in the postpartum period. In several of our CD45 stained images, neutrophils are observed moving through the epithelial barrier towards the uterine lumen. This evidence would indicate that the postpartum uterine inflammation is directed towards the uterine lumen, resulting in an ‘extra-corporeal’ immune response, aimed at limiting inflammation within the endometrial tissue by directing the immune response to the uterine lumen.

Our immunohistochemical analysis also revealed a greater level of disruption to the epithelium in endometritic cows, indicating a role for tissue damage in driving the pathological inflammation associated with endometritis. Tissue damage also has the resulting effect of exposing the underlying stroma, which we found had implications for their role in IL-1β production. Endometrial tissue damage postpartum has classically been attributed to dystocia, defined as a prolonged or difficult calving, often requiring veterinary assistance. Dystocia occurs when the size of the fetus is incompatible with the size of the pelvic opening of the cow. Dystocia trends are increasing internationally, with the rising trend attributed to the introduction of Holstein genes into the dairy herd (Mee 2008). In one study examining Holstein-Friesian dairy cows, the incidence of dystocia was found to increase over a 7 year period from 1997-2004, with cows affected having poorer reproductive performance and decreased milk yield (Gaafar, Shamiah et al. 2011).
In a study of porcine uterine disease, prolonged parturition increased the risk of uterine disease in sows and resulted in delayed uterine involution (Bjorkman, Oliviero et al. 2018). It is clear from our immunohistochemical analysis that tissue damage has major implications for the uterine microenvironment, with the generation of DAMPs and loss of luminal epithelial cell populations and exposure of stromal fibroblasts. Endometrial cells have previously been demonstrated to sense and respond to tissue damage through the production of inflammatory cytokines such as IL-8, IL-6 and low amounts of IL-1α (Healy, Cronin et al. 2014).

Using a model of primary endometrial epithelial cells and stromal fibroblasts we were able to subsequently investigate the contribution of individual endometrial cell populations to production of IL-1 family members and in particular IL-1β. Our previous RNA-seq data had indicated at the expression of a host of IL-1 family members within the endometrium. Epithelial cells and stromal fibroblasts displayed low inducible levels of the IL-1 family members IL1A, IL18 and IL1RN in comparison to PBMCs. Interestingly, stromal fibroblasts displayed strong induction of IL33, while expression of the cytokine was undetectable in either PBMCs or epithelial cells. In the literature, epithelial and endothelial cells are considered the classical producers of IL-33, with limited evidence of IL-33 production by fibroblasts (Moussion, Ortega et al. 2008, Pichery, Mirey et al. 2012, Heyen, Muller et al. 2016). The strong induction of IL33 mRNA by stromal fibroblasts indicate a role for endometrial specific differences in production of IL-1 family cytokines. IL-1β demonstrated the strongest level of induction within our endometrial cultures, confirming our previous RNA-seq data where its elevated expression is associated with uterine disease.

Comparing the level of IL-1β induction observed in PBMCs to what was observed in epithelial cells and stromal fibroblasts, we found that the levels in epithelial cells and PBMCs were comparable, while much higher levels of IL-1β were produced by stromal fibroblasts. Induction of IL-1β at the mRNA level was found to be dependent on LPS stimulation, either alone or in combination with nigericin. Protein expression was dependent on treatment with both LPS and nigericin, with treatment with one stimulus alone having no effect. The requirement for two signals, classically associated with NLRP3 activation, for IL-1β protein production indicates a strong role for inflammasome regulation of IL-1β production within these cells. Levels of IL-1β protein expression in the untreated control samples were also much higher in both epithelial and stromal cells.
than was observed in PBMC controls. These results suggest that both epithelial cells and stromal fibroblasts are primed to respond to inflammatory stimuli and play a role in the production of IL-1β that is comparable or greater than the role of PBMCs. We have demonstrated here that stromal fibroblasts are one of the key producers of IL-1β within the bovine endometrium. The quantities of IL-1β produced by these cells far exceed the quantities produced by PBMCs under the same treatment conditions. As stromal fibroblasts are normally protected from the external environment and uterine microbiome by the overlying epithelial barrier, the loss of this barrier during parturition and the subsequent exposure to the luminal microbiome could result in the elevated expression of IL-1β from stromal fibroblasts, ultimately driving the switch to a pathological inflammatory phenotype in the postpartum endometrium.

Within polarized epithelial cells our results indicate that, while IL-1β is preferentially induced through stimulation at the basolateral membrane, secretion of this cytokine is from the apical membrane, directing the cytokine into the uterine lumen. This is in contrast to our results (section 3.3.4) and other previously published studies which demonstrate that apical stimulation can result in basolateral secretion of cytokines such as IL-8. We hypothesise that the direction of this secretion is linked to the intended function of the cytokine. While IL-8 is directed into the tissue in an attempt to recruit immune cells from the local blood supply, IL-1β is evidently destined to mediate its function within the uterine lumen. As IL-1β has classically been considered to lack a signalling peptide, the mechanism directing its secretion to the apical membrane remains unknown. The implications of this polarized secretion would mean that the postpartum uterine immune responses orchestrated by the epithelial cells are directed into the uterine lumen as opposed to being contained in the uterine tissue. Reports in the literature have previously demonstrated a role for the secretion of host defence peptides by fallopian epithelial cells into the uterine lumen and for the secretion of cytokines such as IL-6 into the lumen by endometrial epithelial cells (Ghosh, Schaefer et al. 2008, Healy, Cronin et al. 2015). Previous work by our group has also demonstrated that IL-1β is detectable in cervical-vaginal mucus postpartum, indicating that it is secreted into the lumen and exits the uterus as a component of mucus (Adnane, Chapwanya et al. 2017). The ability of epithelial cells, and inability of stromal fibroblasts, to directionally secrete IL-1β has a number of implications for the cytokine’s role in uterine disease. Directing secreted IL-1β into the uterine lumen ensures that the cytokine is localized to the primary site of
infection, ready to activate neutrophils recruited to the uterine lumen. In contrast, the undirected release of IL-1β by stromal fibroblasts has the potential to propagate the inflammatory response within the endometrial tissue.

Analysis of IL-1β production by Western blotting highlighted a number of key issues. As previously observed in bovine PBMCs, unstimulated endometrial epithelial cells and stromal fibroblasts contain pre-formed IL-1β which can be released from the cell in response to nigericin alone. This has previously been reported to be a bovine specific response (Harte, Gorman et al. 2017). The presence of this pre-formed IL-1β in bovine cells might also account for the fact that while only basolateral stimulation resulted in IL-1β mRNA induction, both apical and basolateral stimulation resulted in the release of IL-1β protein. Additionally, a higher molecular weight isoform of IL-1β was present in epithelial cells in this study. Within the literature, only one study has reported a high molecular weight form of IL-1β, which, interestingly from a reproductive angle, was found in human amniotic fluid (Tamatani, Tsunoda et al. 1988). While this study was able to identify the presence of this IL-1β variant in amniotic fluid, the nature of this variant was not identified.

Given the directional secretion of IL-1β observed in our polarized epithelial cells, we hypothesized that the high molecular weight isoform of IL-1β observed in epithelial cells is due to a signal peptide, responsible for directing the cytokine to the apical membrane. We investigated a number of possible modifications, both transcriptional and post-translational mechanisms, but were unable to identify the mechanism behind IL-1β modification in epithelial cells. While the events surround IL-1β processing have been well documented, there has been much debate in the literature surrounding the mechanism of IL-1β secretion. IL-1β lacks a signal sequence to direct its exit from the cell via secretory vesicles and so must follow an unconventional secretory pathway. The current model for IL-1β secretion from the cell involves caspase-1 cleavage of gasdermin D, which in turn forms pores in the cell membrane causing pyroptotic cell death, with IL-1β released during cell lysis (Cullen, Kearney et al. 2015, Aglietti, Estevez et al. 2016, Liu, Zhang et al. 2016). A recent study has identified a polybasic motif within the active form of the human and mouse IL-1β cytokine which directs the mature cytokine to ruffles in the plasma membrane where gasdermin D forms pores allowing for cytokine release (Monteleone, Stanley et al. 2018). It is possible that the higher molecular weight variant of IL-1β contains a similar motif, directing IL-1β exclusively to the apical membrane.
within epithelial cells. While we hypothesized that the variant is associated with release from the cell, there is also the possibility that the variant is associated with IL-1β activity following release from the cell and has no function in directing the cytokine for release. Higher molecular weight variant may be designed to have different effect on its cellular targets, either binding more or less efficiently to its cognate receptor. Future work must address this and so it is crucial to isolate and sequence this variant in order to synthesise a recombinant protein to allow for functional assays.

IL-1β has previously been shown to affect tight junction proteins in human intestinal and bovine mammary gland epithelial cells, resulting in permeability of the epithelial barrier (Al-Sadi and Ma 2007, Xu, Dong et al. 2018). Thus, we hypothesized that the undirected production of IL-1β by stromal fibroblasts has the potential to limit the ability of epithelial cells to regulate inflammation. Our results indicate that basolateral treatment of an established epithelial barrier with increasing concentrations of IL-1β had no effect on barrier permeability. However, when the epithelial tight junctions are disrupted and allowed to re-establish, similar to what is occurring postpartum, treatment with IL-1β significantly affected the ability of the epithelial barrier to recover. The ability of IL-1β to delay barrier recovery was similar whether treatment was administered apically or basolaterally. Interestingly, when treated basolaterally, higher concentrations of IL-1β had less of an effect on epithelial barrier recovery than lower concentrations. A similar observation had previously been reported in intestinal epithelial cells treated with increasing concentrations of IL-1β. Treatment with 10 ng/ml IL-1β resulted in the maximum reduction in TER values, with increasing concentrations (50-100 ng/ml) having less of an impact on TER values (Al-Sadi and Ma 2007). While our results here simply demonstrate the pathological effect IL-1β has on the regenerating epithelium, further work will be needed to examine the mechanism behind this inhibition of epithelial barrier regeneration. In studies with intestinal and bovine mammary epithelial cells, IL-1β was found to mediate its effects on epithelial barrier disruption by NFκB dependent increase in protein expression of the myosin L chain kinase (MLCK) which can mediate the opening of the tight junction barrier (Al-Sadi, Ye et al. 2008, Xu, Dong et al. 2018). The ability of IL-1β to delay epithelial barrier recovery represents an important mechanism in the switch to pathological inflammation, has implications for mechanisms of tissue repair and involution in the postpartum uterus and represents a possible route for IL-1β in driving its own dysregulated expression. By delaying epithelial cell
regeneration, stromal fibroblasts are exposed to a continuous supply of luminal antigens, DAMPs and other toxic by-products, thereby mounting and enhancing the inflammatory response postpartum.

Our work here examining the expression of IL-1β within the endometrium has given us a greater understanding of the events surrounding the switch from a healthy to a pathological inflammatory immune response postpartum and has demonstrated novel roles for epithelial cells and in particular stromal fibroblasts in mediating this inflammation (Figure 4.29). During a healthy inflammatory response, IL-1β is released from the apical epithelial membrane into the uterine lumen. The IL-1β present in the lumen activates the immune cells which have been recruited, initiating an ‘extra-corporeal’ immune response. During a pathological inflammatory immune response postpartum, disruption to the epithelial barrier results in the exposure of the underlying stromal fibroblasts and the basolateral membrane of the epithelium to the uterine microflora resulting in the amplification of IL-1β expression and the luminal leakage of this potent inflammatory cytokine. While our work here has focused on the role of IL-1β, its production remains one of the many possible mechanisms driving the switch from physiological to pathological inflammation. Factors such as the metabolic and health status of the cow, difficulty calving etc. can all contribute to the pathological switch associated with endometritis and may pre-date the production of IL-1β. Further work is needed to thoroughly investigate the contribution of these factors to the switch to pathology.
Figure 4.29. Proposed model of healthy and pathological inflammation in the postpartum endometrium.

Model detailing the events surrounding postpartum uterine inflammation (either healthy or pathological). (A). A healthy (or adaptive) inflammatory response in the postpartum endometrium is characterised by a (mostly) intact epithelium, IL-1β production by the epithelial cells and its subsequent release into the uterine lumen. (B). A pathological inflammatory immune response is characterised by a disrupted epithelium resulting in the exposure of the stromal fibroblasts. IL-1β production occurs in the exposed stromal fibroblasts, resulting in the undirected secretion of IL-1β into both the lumen and surrounding endometrial tissue, propagating and prolonging the period of pathological inflammation.

Given the role of IL-1β in endometritis pathology, targeting production of IL-1β might be of therapeutic benefit. In order to achieve this, we need a greater understanding of the regulatory mechanisms surround the cleavage of IL-1β into its active form and its subsequent release from the cell.
Chapter 5 Inflammasome component expression and function in the bovine endometrium

5.1 Introduction

The inflammasome signalling complex has been identified as the major regulator of IL-1β release from the cell, responsible for mediating the cleavage of pro-IL-1β into its active form (Martinon, Burns et al. 2002). Since its initial discovery, the inflammasome family has expanded to include the nod-like receptor family of inflammasomes composed of NLRP1B, NLRP3 and NLRC4 and the pyhin family of inflammasomes which includes AIM2 and IFI16. Several other less characterized inflammasomes exist including the NLRP6, NLRP7, NLRP12 and RIG-I inflammasomes (Sims and Smith 2010). Extensive research has been undertaken to elucidate the mechanisms and roles of inflammasome activation, with the majority of this research being undertaken in mouse models of inflammatory disease.

Previous work by our laboratory has reported that the NLRP3 receptor demonstrated the highest expression of the inflammasome receptors in the postpartum endometrium (Foley, Chapwanya et al. 2015). The canonical NLRP3 inflammasome complex is composed of the receptor NLRP3, the adaptor protein ASC and the effector caspases which are recruited and ultimately mediate the cleavage of IL-1β into its active form. Inflammasome activation typically requires two signals, the first in the form of a PAMP such as LPS which signals through the TLR pathway and results in the production of pro-IL-1β. The second signal is the inflammasome activatory signal and comes in many different forms, ranging from microbial toxins such as nigericin, DAMPs such as extracellular ATP to markers of metabolic stress such as extracellular glucose and monosodium urate crystals (Perregaux, Barberia et al. 1992, Schroder and Tschopp 2010). The net effect of these stimuli is potassium efflux from the cell which has been found to be essential for activation of the NLRP3 receptor (Petrilli, Papin et al. 2007).

Activation of the NLRP3 receptor results in its oligomerisation promoted by NEK7 (He, Zeng et al. 2016). Oligomerisation of NLRP3 subsequently results in the recruitment of the adaptor protein ASC, which interacts with NLRP3 through its PYD domain. ASC also contains a CARD domain which it uses to interact with the same domain of Caspase-
1, recruiting it to the inflammasome (Mariathasan and Monack 2007). The inflammasome then cleaves pro-caspase-1 into its active form, resulting in the formation of active IL-1β. Caspase-1 also mediates the cleavage of gasdermin D into its active form, allowing it to travel to the cell membrane and form pores, resulting in pyroptotic cell death and permitting IL-1β release (Figure 5.1).

Figure 5.1. The canonical NLRP3 inflammasome signalling pathway.

Following LPS priming through the TLR pathway, the NLRP3 receptor will respond to stimuli such as ATP or nigericin. This results in NLRP3 oligomerisation (promoted by NEK-7) and recruitment of the ASC adaptor complex. ASC in turn mediates the cleavage of pro-caspase-1 into its active form. Active caspase-1 can then mediate the cleavage of pro-IL-1β into its active form, allowing its release from the cell. Active caspase-1 also mediates the cleavage of gasdermin D into its active form, which in turn travels to the cell membrane, forming pores and resulting in pyroptotic cell death.

Inflammasome activation in mouse and human has been the main focus of inflammasome research to date, with the majority of research being performed on human or murine monocytes or macrophages. Comparative research into bovine specific mechanisms of
inflammasome signalling has received significantly less attention. Given the evolutionary differences that exist across human, mouse and bovine species, mechanisms observed in human and mouse may not necessarily apply to bovine. Species specific differences in inflammasome operation have already been identified in the bovine, with the genes encoding the AIM2 inflammasome found to be pseudogenised in the bovine (Cridland, Curley et al. 2012). Evidence is also emerging demonstrating non-canonical inflammasome pathways operating in what are considered ‘non-professional’ immune cells such as epithelial cells, keratinocytes and fibroblasts at various tissue sites (Crowley, Vallance et al. 2017). In comparison to the canonical pathway of inflammasome activation (Figure 5.1), the species, tissue or cell specific differences resulting in an alternative or non-canonical pathways have received relatively little attention.

The term non-canonical inflammasome had initially been coined to describe inflammasome signalling involving caspase-11 in mice (Kayagaki, Warming et al. 2011). This non-canonical pathway originally described a process whereby cytoplasmic LPS is sensed by murine caspase-11 and its human orthologs caspase-4 and caspase-5, triggering the process of pyroptosis while also activating the NLRP3 inflammasome, resulting in the downstream cleavage of capase-1 and subsequent activation of IL-1β and IL-18. Since its initial discovery surrounding the events of caspase-11 activation of the NLRP3 inflammasome in mice, the term non-canonical inflammasome has since grown to include any modification to the canonical pathway utilising caspase-1 mediated cleavage of IL-1β.

While the role for caspase-1 in IL-1β processing has been well defined, the involvement of other inflammatory caspases, such as caspase-8, in inflammasome signalling is only beginning to emerge. Work by Chi et al (2014) has demonstrated a role for caspase-8 in activating both the NLRP1 and NLRP3 pathways following TLR4 activation. Increased expression of caspase-8 following TLR4 activation resulted in increased IL-1β expression (Chi, Li et al. 2014). In the bovine, alum induced secretion of IL-1β from PBMCs has also been shown to be caspase-8 dependent (Harte, Gorman et al. 2017). A role for caspase-4 in mediating the cleavage of IL-18, independently of caspase-1, has been demonstrated in human intestinal epithelial cells (Knodler, Crowley et al. 2014).

Recently, evidence has been emerging of species specific differences existing between the mechanism of inflammasome activation in mice and humans. Gaidt et al (2016) have
shown that human monocytes do not engage the classical inflammasome pathway as has been characterised in mice, instead engaging an alternative pathway that still involves the use of classical inflammasome components such as NLRP3, ASC and caspase-1. However this alternative pathway did not display any of the classical characteristics of inflammasome activation including the requirement for potassium efflux to initiate inflammasome activation, pyroptosome formation and subsequent pyroptosis (Gaidt, Ebert et al. 2016). Wang et al (2013) have also demonstrated that signal one alone (signalling though the TLR receptor) is sufficient to induce inflammasome activation in human monocytes, in contrast to the two-signal model established in the mouse (Wang, Mao et al. 2013).

Reports have demonstrated that similar to the human and mouse, the bovine inflammasome controls the release of IL-1β in response to classical inflammasome stimuli such as ATP in bovine monocytes (Hussen, Düvel et al. 2012). However, despite the similarities, some specific differences also exist. Bovine monocytes do not require the ATP-gated ion channel P2X7 for inflammasome activation despite requiring potassium efflux as the second signal for inflammasome activation (Hussen, Düvel et al. 2012). Interestingly, recent evidence demonstrated bovine breed specific differences in IL-1β production between the high milk yielding dairy cow Holstein-Friesian breed and the Brown Swiss beef breed (Gibson, Woodman et al. 2016). Bovine PBMCs have been reported to contain pre-formed IL-1β which can be released from the cell in response to treatment LPS alone or the inflammasome activating agent alum alone, which was not observed in human PBMCs treated with the same conditions. Bovine PBMCs also demonstrated levels of active caspase-1 in untreated cells, a phenomenon not observed in human PBMCs (Harte, Gorman et al. 2017).

Here we aimed to look at inflammasome activation in bovine endometrial cells as a possible explanation for the raised levels of IL-1β we observed in endometritic cows. Elucidating the regulatory pathways surrounding IL-1β release in endometrial cells will allow for a better understanding of the mechanisms surrounding the switch to a pathological inflammatory response in the postpartum endometrium. Based on descriptions of species-, tissue- and cell-specific differences surrounding the activation and mechanism of action of the NLRP3 inflammasome, it was necessary to examine the expression of components and activation of inflammasome pathways operating in bovine endometrial cells. We aimed to examine the conservation of inflammasome components.
within the bovine genome and bioinformatically examine the similarities in protein functional regions across the human, mouse and bovine. Through the use of inhibitors targeting specific inflammasome components including the NLRP3 receptor and selected inflammatory caspases, we aimed to determine whether the IL-1β secretion we have previously observed in endometrial cells is inflammasome dependent or is being secreted through an alternative pathway.

5.2 Hypothesis and specific aims

We hypothesise that IL-1β production in endometrial epithelial and stromal fibroblast cells is dependent on the activation of the NLRP3 inflammasome signalling pathway for its cleavage and release from the cell and that potential species or cell specific differences in inflammasome signalling in the bovine endometrium may exist.

The specific aims were:

1. Investigate the presence and conservation of inflammasome components within the bovine genome.
2. Examine the basal mRNA expression and mRNA induction of inflammasome components, including the NLRP3 receptor, NEK-7, the adaptor ASC, gasdermin D and caspase -1 and -4 involved in IL-1β cleavage.
3. Determine if the IL-1β being produced by endometrial cells is inflammasome dependent through the use of NLRP3 and caspase specific inhibitors.
5.3 Results

5.3.1 Inflammasome components are conserved across species

Given the discrepancies that exist between the mechanisms of inflammasome activation between human and mouse, it was important to first determine the presence and level of conservation of canonical NLRP3 inflammasome components within the bovine. We utilised publicly available bioinformatic tools to characterise the conservation of the NLRP3 receptor, the adaptor protein ASC and caspase-1 within the bovine genome, examine the level of homology between bovine, human and mouse inflammasome protein components and determine the conservation of the functional protein domains of NLRP3 inflammasome components across human, mouse and bovine. Bioinformatic analysis of the presence and level of conservation of these components across species is a crucial first step in identifying any possible discrepancies in existing in the NLRP3 signalling pathway and may better inform future investigations into the relative contribution of individual inflammasome components to IL-1β secretion.

The gene encoding the NLRP3 receptor is present and annotated within human, mouse and bovine genomes (Figure 5.2 A.). Within the human, NLRP3 is located on chromosome 1, is 49,430 base pairs and is composed of 11 exons. Within the mouse genome, the NLRP3 gene is located on chromosome 11, is 36,407 base pairs and similar to the human is composed of 11 exons. Within the bovine genome, the NLRP3 gene is located on chromosome 7, is similar in length to the human gene being 49,736 base pairs and consists of 11 exons.

The gene encoding the adaptor protein ASC was also found to be present and annotated in the human, mouse and bovine genomes (Figure 5.2 B.). Within the human genome, ASC is located on chromosome 7, is 1487 base pairs and consists of 3 exons. Within the mouse genome ASC (located on chromosome 16) is of similar length, consisting of 1,937 base pairs and composed of 3 exons. Similarly, within the bovine genome ASC (located on chromosome 25) is similar to both human and mouse, consisting of 2,000 base pairs and is composed of 3 exons.

Caspase-1 exhibits the most variation across genomes, although still present and annotated across all genomes examined here (Figure 5.2 C.). Within the human genome
it is located on chromosome 11, is 9,648 base pairs and consists of 9 exons. In the mouse genome, caspase-1 is located on chromosome 9, is 8,118 base pairs and consists of 10 exons. Within the bovine genome caspase-1 is located on chromosome 15, is 14,243 base pairs and consists of 8 exons.

**Figure 5.2. The genes for the inflammasome components NLRP3, ASC and caspase-1 are conserved across the human, mouse and bovine genomes**

Examining the conservation of the genes encoding (A.), the NLRP3 receptor, (B.), the adaptor protein ASC and (C.), caspase-1 across the human (*Homo Sapiens*), mouse (*Mus musculus*), and bovine (*Bos taurus*) genome. Inflammasome components were searched for within their respective genomes using the publicly available UCSC genome browser. Gene images were obtained from UCSC genome browser with exons indicated as thick shaded boxes and introns as thin crossed lines.

Having identified conservation of the NLRP3 inflammasome gene components within the bovine genome, we next wanted to examine the level of protein homology that exists across human, mouse and bovine. Sequence similarity searches using freely available programmes such as BLAST are capable of identifying homologous proteins using statistically significant similarities that reflects common ancestry (Pearson 2013). Using a BLAST search gives a number of readouts, namely the percentage identity, the E-value and the query cover. The percentage identity quantifies the extent to which two amino acid sequences have the same residues in the same position. The query cover describes the percentage of the query sequence that is aligned. The E (expect)-value describes the
number of expected hits a sequence might get based on random chance. The lower the E-value/closer to zero, the more significant the match.

Examining the similarity of the NLRP3 receptor across species (Table 5.1), we see a high level of protein homology between bovine and human (83% identity) and between bovine and mouse (79% identity). Examining similarity of the ASC adaptor complex, we see a similar high level of homology between bovine and human (72% identity) and bovine and mouse (74% identity). While caspase-1 demonstrates the lowest level of homology of the inflammasome components examined, there is still a relatively high level of similarity between bovine and human (68% identity) and bovine and mouse (64% identity). Based on the high level of similarity among inflammasome components across species, we can infer that the bovine components are likely orthologs of their human and mouse counterparts and that a high level of homology exists in the NLRP3 inflammasome pathway across species. Query cover is high for all three components examined indicating a strong alignment and the E-value remains close to zero indicating a high level of statistical significance between the observed alignments.

Table 5.1. Identifying inflammasome component protein homology across species.

<table>
<thead>
<tr>
<th>Component</th>
<th>Query Cover</th>
<th>E-value</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLRP3 (NP_001095689.1)</td>
<td>99%</td>
<td>0.0</td>
<td>83%</td>
</tr>
<tr>
<td>Homo Sapiens (NP_001073289.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mus Musculus (NP_665826.1)</td>
<td>99%</td>
<td>0.0</td>
<td>79%</td>
</tr>
<tr>
<td>ASC (NP_777155.1)</td>
<td>100%</td>
<td>2e-92</td>
<td>72%</td>
</tr>
<tr>
<td>Homo Sapiens (NP_660183.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mus Musculus (NP_075747.3)</td>
<td>100%</td>
<td>1e-100</td>
<td>74%</td>
</tr>
<tr>
<td>Caspase-1 (XP_002692967.1)</td>
<td>100%</td>
<td>0.0</td>
<td>68%</td>
</tr>
<tr>
<td>Homo Sapiens (NP_001214.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mus Musculus (NP_033937.2)</td>
<td>100%</td>
<td>0.0</td>
<td>64%</td>
</tr>
</tbody>
</table>

Having observed a relatively high level of protein homology of inflammasome components across human, mouse and bovine, we next wanted to examine the similarities in their protein structure and in particular, the conservation of the functional domains of these proteins across species. Using a publically available protein analysis tool, InterPro, we examined the conservation of functional components of the inflammasome proteins.
across the human, mouse and bovine. The InterPro protein analysis tool is a free resource that provides functional analysis of protein sequences by classifying them into families and predicting the presence of functionally important domains (Finn, Attwood et al. 2017). To achieve this InterPro uses predictive models combined from multiple different databases.

Examining the conservation of functional components of the NLRP3 receptor across human, mouse and bovine (Figure 5.3), we see that the three receptors appear structurally identical with all three species containing a pyrin domain, a NACHT associated domain, a P-loop containing nucleoside triphosphate hydrolase domain and a leucine rich repeat domain. All identified components had previously been identified in the literature as being required components for NLRP3 receptor function (Mariathasan and Monack 2007). The NLRP3 receptor was also similar in size across human (1,016 amino acids), mouse (1,033 amino acids) and bovine (1,031 amino acids).
**Figure 5.3. NLRP3 protein domains are conserved across species.**

Comparison of functional domains of the NLRP3 receptor in human (*Homo sapiens*), mouse (*Mus musculus*) and bovine (*Bos taurus*). For structural domain analysis, FASTA sequences of the proteins of interest were submitted to the InterPro v54 sequence, analysis and classification server (http://www.ebi.ac.uk/interpro/). InterPro predicts the presence of functionally important domains using predictive models provided by several different databases.

Next we examined the conservation of functional components of the ASC adaptor complex across human, mouse and bovine (Figure 5.4). We observed that the three adaptor complexes appear structurally identical with all three species containing a pyrin death-like domain and a CARD domain. All identified components had previously been described in the literature as being required components for ASC function, with the pyrin domain required for interaction with the NLRP3 receptor and the CARD domain required to recruit and interact with the CARD domain on caspase-1 (Mariathasan and Monack 2007). The ASC adaptor complex was also similar in size across human (176 amino acids), mouse (193 amino acids) and bovine (195 amino acids).
**Figure 5.4. ASC protein domains are conserved across species.**

Comparison of functional domains of the ASC adaptor complex in human (*Homo sapiens*), mouse (*Mus musculus*) and bovine (*Bos taurus*). For structural domain analysis, FASTA sequences of the proteins of interest were submitted to the InterPro v54 sequence, analysis and classification server (http://www.ebi.ac.uk/interpro/). InterPro predicts the presence of functionally important domains using predictive models provided by several different databases.

We also examined the conservation of functional components of caspase-1 across human, mouse and bovine (Figure 5.5). Despite the differences in gene structure observed across the three species, caspase-1 appears structurally identical at the protein level with all three species containing a CARD domain, a caspase-like domain and peptidase C14 caspase subunit. These components had previously been identified in the literature as being required for caspase-1 function, with the CARD domain required for interaction with the CARD domain on the ASC adaptor complex and the caspase domain required for IL-1β cleavage into its active form (Bouchier-Hayes, Conroy et al. 2001). Caspase-1 was also similar in size across human (383 amino acids), mouse (402 amino acids) and bovine (403 amino acids).
Figure 5.5. Caspase-1 protein domains are conserved across species.

Comparison of functional domains of the caspase-1 in human (*Homo sapiens*), mouse (*Mus musculus*) and bovine (*Bos taurus*). For structural domain analysis, FASTA sequences of the proteins of interest were submitted to the InterPro v54 sequence, analysis and classification server (http://www.ebi.ac.uk/interpro/). InterPro predicts the presence of functionally important domains using predictive models provided by several different databases.

Given the high levels of gene conservation, protein homology and conservation of functional protein domains of the inflammasome components across the three species examined here, we are confident that the components for the NLRP3 inflammasome signalling pathway are preserved in the bovine. We next needed to determine the induction and expression of these inflammasome components within bovine cells and elucidate the exact signalling mechanism utilised by the NLRP3 inflammasome pathway within the bovine and in particular, within the bovine endometrium.
5.3.2 Inflammasome components are expressed in bovine endometrial cells

Having previously established that endometrial epithelial cells and stromal fibroblasts are potent producers of IL-1β and given the limited data on inflammasome signalling pathways within endometrial cells and within the bovine, we explored expression of the relevant NLRP3 inflammasome components in endometrial cells compared to circulating immune cells, which are considered to be the classical producers of IL-1β. We began by examining the baseline expression and subsequent induction of genes encoding inflammasome components such as NEK-7, the NLRP3 receptor, the ASC adaptor complex, gasdermin D and caspases involved in inflammasome signalling following a time course stimulation over 24 hours with the inflammasome priming PAMP LPS and the inflammasome activator nigericin. Constitutive mRNA levels were examined in the un-stimulated samples. mRNA induction was examined in response to a time course stimulation with LPS (2 µg/ml) and nigericin (10 µM).

Baseline expression of *NLRP3* in epithelial cells was higher than PBMCs or stromal fibroblasts, where levels were comparable (1.3×10⁻¹ relative expression units to *H3F3A* versus 0.7×10⁻¹ in PBMCs and 0.5×10⁻¹ in stromal fibroblasts) (**Figure 5.6 A.**). Baseline expression of *NEK7* was significantly higher in PBMCs (6.6×10⁻² relative expression units to *H3F3A*) compared to epithelial cells (0.7×10⁻²; p=0.0459) and stromal fibroblasts (1.9×10⁻²; p=0.0303) (**Figure 5.6 B.**). Baseline levels of the gene encoding the adaptor protein ASC were significantly higher in PBMCs (2.1×10⁰ relative expression units to *H3F3A*) compared to epithelial cells (0.1×10⁰; p=0.0002), and were also significantly higher in stromal fibroblasts (0.4×10⁰; p=0.0145) compared to epithelial cells (**Figure 5.6 C.**). Gasdermin D was expressed across stromal fibroblasts (2.5×10⁻² relative expression units to *H3F3A*), PBMCs (1.2×10⁻²) and epithelial cells (0.4×10⁻²) (**Figure 5.6 D.**).

Interestingly, while *caspase-1* was expressed in PBMCs (4.2×10⁻³ relative expression units to *H3F3A*), *caspase-1* expression could not be detected in endometrial cells (**Figure 5.6 E.**). This was despite the fact that we designed and utilised a number of different primer sets, with each set targeting a distinct region of the *caspase-1* gene. *Caspase-1* was also absent from our RNA-seq dataset comparing healthy and endometritic cows at 7 and 21 DPP (Foley, Chapwanya et al. 2015). The absence of *caspase-1* expression in endometrial cells is a strong indicator of a potential anomaly in the NLRP3 signalling
pathway within bovine endometrial cells. The expression and role of inflammatory caspases in inflammasome mediated cleavage of IL-1β in endometrial cells was subsequently examined in greater detail (detailed in Section 5.3.5). However, having observed a lack of caspase-1 expression within endometrial cells, we probed our RNA-seq dataset for an alternative caspase enzyme that might be involved in IL-1β cleavage. Caspase-4 was significantly differentially expressed between healthy and endometritic cows and a number of previous studies had previously identified its role in non-canonical inflammasome signalling and so we aimed to examine its expression in our endometrial cell populations. We identified significantly higher baseline expression of caspase-4 in PBMCs (5.6×10^{-1} constitutive expression relative to H3F3A) compared to stromal fibroblasts (0.9×10^{-1}). Baseline expression levels were also higher in epithelial cells (2.3×10^{-1}) compared to stromal fibroblasts (Figure 5.6 F.).
Figure 5.6. Endometrial cell populations display different baseline expression levels of the NLRP3 inflammasome signalling components. 

(A). NLRP3, (B). NEK7, (C). ASC, (D). gasdermin D, (E). caspase-1 and (F). caspase-4 mRNA baseline expression relative to H3F3A across unstimulated samples. Cells were harvested in TRIzol. RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene H3F3A. Columns indicate mean expression relative to H3F3A, error bars indicate standard error of the mean (n=5). Statistical analysis was performed using a Kruskall Wallis test with Dunns multiple comparison test. *=p≤0.05; **=p≤0.001; ***=p≤0.0001.
We next examined induction of the NLRP3 inflammasome components in PBMCs, epithelial cells (either 2D culture or polarized) and stromal fibroblasts in response to a time-course stimulation with the inflammasome priming signal LPS and the inflammasome activating agent nigericin over a period of 24 hours. Induction of the specific inflammasome components demonstrated distinct expression patterns in each of the cell types examined.

Expression of NLRP3 was induced across PBMCs (MFC of 12.6) and stromal fibroblasts (MFC of 10.4) 6 hours post stimulation (Figure 5.7 A. & D.). NLRP3 peaked earlier in 2D stimulated epithelial cells with a MFC of 3.9 at 3 hours post stimulation (Figure 5.7 B.). NLRP3 was significantly elevated in polarized epithelial cells at 3 hours post stimulation with a MFC of 7.8 and 6.4 (p=0.0079) following basolateral and apical stimulation respectively (Figure 5.7 C.), although expression was not significantly different between apical or basolateral stimulation.
Figure 5.7. NLRP3 is induced in endometrial cells in response to LPS and the inflammasome activating agent nigericin.

NLRP3 mRNA fold change following stimulation in (A.) PBMCs, (B.) 2D cultured epithelial cells, (C.) polarized epithelial cells and (D.) stromal fibroblasts. Following stimulation with LPS and nigericin, cells were harvested in TRIzol. RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene H3F3A. Columns indicate mean fold change relative to unstimulated control, error bars indicate standard error of the mean (n=5). Statistical analysis was performed using a Kruskal Wallis test with Dunns multiple comparison test. **=p≤0.001.

Induction of NEK7, the gene encoding the protein required for NLRP3 oligomerisation, was detected in stromal fibroblasts 3 hours post stimulation with a MFC of 6.7 (Figure 5.8 D.). NEK7 was constitutively expressed across PBMC (Figure 5.8 A.) and epithelial cell stimulations (Figure 5.8 B.) and peaked significantly in polarized epithelial cells following 12 hours of basolateral stimulation with a MFC of 3.2 (p=0.0079) (Figure 5.8 C.).
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Figure 5.8. Endometrial cells display differential induction of *NEK7* mRNA.

*NEK7* mRNA fold change following stimulation in (A.). PBMCs, (B.). 2D cultured epithelial cells, (C.). polarized epithelial cells and (D.). stromal fibroblasts. Following stimulation with LPS and nigericin, cells were harvested in TRizol. RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene *H3F3A*. Columns indicate mean fold change relative to unstimulated control, error bars indicate standard error of the mean (*n*=5). Statistical analysis was performed using a Kruskal Wallis test with Dunns multiple comparison test. **=*p*≤0.001.

The gene encoding the adaptor protein ASC displayed constitutive expression across PBMC, epithelial and stromal fibroblasts (Figure 5.9 A., B. & D.). However, polarized epithelial cells displayed a peak in expression 6 hours post stimulation with a MFC of 2.9 and 2.3 following basolateral and apical stimulation respectively and differential expression 24 hours post stimulation, with higher expression observed following apical stimulation with a MFC of 2.2 compared to a MFC of 0.5 following basolateral stimulation (Figure 5.9 C.).
Figure 5.9. Levels of ASC mRNA induction appear constant across endometrial cell populations.

ASC mRNA fold change following stimulation in (A.), PBMCs, (B.). 2D cultured epithelial cells, (C.), polarized epithelial cells and (D.). stromal fibroblasts. Following stimulation with LPS and nigericin, cells were harvested in TRIzol. RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene H3F3A. Columns indicate mean fold change relative to unstimulated control, error bars indicate standard error of the mean ($n=5$). Statistical analysis was performed using a Kruskal Wallis test with Dunns multiple comparison test.

Gasdermin D expression was low across PBMC (peak MFC of 2.5 at 12 hours post-stimulation), epithelial cells (peak MFC of 2.5 at 3 hours post-stimulation) and stromal fibroblasts (peak MFC of 2.3 at 3 hours post-stimulation) (Figure 5.10 A., B. & D.). Polarized epithelial cells displayed the highest level of expression following apical stimulation at 6 (MFC of 4.6 compared to a MFC of 0.8 following basolateral stimulation) and 12 (MFC of 4.6 compared to a MFC of 3.5 following basolateral stimulation) hours post stimulation (Figure 5.10 C.).
Figure 5.10. *Gasdermin D* levels are not strongly induced in endometrial cell populations.

*Gasdermin D* mRNA fold change following stimulation in (A.). PBMCs, (B.). 2D cultured epithelial cells, (C.). polarized epithelial cells and (D.). stromal fibroblasts. Following stimulation with LPS and nigericin, cells were harvested in TRIzol. RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene *H3F3A*. Columns indicate mean fold change relative to unstimulated control, error bars indicate standard error of the mean (n=5). Statistical analysis was performed using a Kruskal Wallis test with Dunns multiple comparison test.

Caspase-4 expression was significantly elevated in PBMCs (with a MFC of 9.7; p=0.0079) (Figure 5.11 A.) and apically stimulated polarized epithelial cells (with a MFC of 9.1; p=0.0286) following 24 hours of stimulation (Figure 5.11 C.). Expression in 2D cultured epithelial cells peaked earlier at 6 hours post stimulation with a significant MFC of 6.1 (p=0.0079) (Figure 5.11 B.), while stromal fibroblasts displayed constitutive expression across all time points (Figure 5.11 D.).
Figure 5.11. Endometrial cells display differential induction of *caspase-4* mRNA.

*Caspase-4* mRNA fold change following stimulation in (A.). PBMCs, (B.). 2D cultured epithelial cells, (C.). polarized epithelial cells and (D.). stromal fibroblasts. Following stimulation with LPS and nigericin, cells were harvested in TRIzol. RNA was extracted and first strand cDNA reverse transcribed. qPCR was carried out and gene Cq values were normalised to the reference gene *H3F3A*. Columns indicate mean fold change relative to unstimulated control, error bars indicate standard error of the mean (*n*=5). Statistical analysis was performed using a Kruskal Wallis test with Dunns multiple comparison test. *=p≤0.05; **=p≤0.001.

These results suggest that similar to studies performed in the human and mouse, components of the NLRP3 inflammasome are induced in endometrial cells in response to inflammatory stimuli. Using the knowledge that these NLRP3 inflammasome components are induced in response to the inflammasome activating signals LPS and nigericin, we can now elucidate the inflammasome signalling pathway operating within bovine endometrial cells through the use of inhibitors targeting specific points of this pathway.
5.3.3 Inflammasome activity in endometrial cells is dependent on the NLRP3 receptor

Previous reports from both mouse and human studies have demonstrated that IL-1β production is dependent on the NLRP3 inflammasome receptor and the inflammatory caspases for mediating IL-1β cleavage and release from the cell (Martinon, Burns et al. 2002). Given the evidence presented previously, supporting a role for epithelial cell and stromal fibroblast mediated IL-1β production in the endometrium and having demonstrated mRNA induction of specific inflammasome components, we aimed to determine if IL-1β production was dependent on the inflammasome complex for its release using inhibitors targeting specific inflammasome components. This represents the first study to examine the role of the NLRP3 receptor and inflammatory caspases in mediating IL-1β release in bovine endometrial cells.

Using a recently developed small molecule inhibitor specific to the NLRP3 receptor in humans and mice, we aimed to investigate the contribution of the NLRP3 receptor to IL-1β production in bovine endometrial cells by blocking its activity and measuring IL-1β secretion. The small molecule inhibitor termed MCC950 has been reported as a highly potent inhibitor, capable of blocking both canonical and non-canonical NLRP3 activation (Coll, Robertson et al. 2015). In addition, this small molecule inhibitor was found to specifically inhibit the action of the NLRP3 receptor but had no impact on the AIM2, NLRC4 or NLRP1 inflammasome receptor. MCC950 significantly reduced IL-1β levels in-vivo and attenuated the severity of EAE, a mouse model of multiple sclerosis. Given the success of this inhibitor in-vivo, it has been identified as a potential therapeutic for NLRP3 associated inflammatory diseases in humans. We aimed to utilise this inhibitor to investigate the contribution of the NLRP3 inflammasome receptor to IL-1β secretion in the endometrium.

A number of preliminary experiments were performed in order to optimise the conditions of MCC950 treatment. The point at which the inhibitor was added to cells was initially examined, with cells treated prior to the 6 hours of LPS priming or prior to the addition of the inflammasome activating agent nigericin for 1 hour. We found that while addition of the same increasing concentration of MCC950 before stimulation with nigericin resulted in inhibition of IL-1β expression in both cell populations compared to the positive controls, the level of inhibition observed was not the same as had been observed
with adding the inhibitor before LPS priming. While concentrations of 100-1,000 nM MCC950 had proven effective in human PBMCs and concentrations of 100-10,000 nM had proven effective in murine BMDMs, we initially treated cells with a wide range of inhibitor concentrations in order to define the optimal concentrations. Additionally, epithelial cells were polarized prior to treatment, with stimulation and inhibitor treatment occurring at the basolateral membrane while supernatants were harvested from the apical compartment which we had previously demonstrated resulted in optimal IL-1β secretion. Stromal fibroblasts were treated in 2D culture.

As we have previously demonstrated, treatment of endometrial epithelial cells and stromal fibroblasts with LPS and nigericin for 6 hours resulted in a dramatic increase in IL-1β secretion from both cell populations (Figure 5.12). Treatment of endometrial epithelial cells and stromal fibroblast cells with increasing concentrations of MCC950 (1-100 nM) prior to LPS priming resulted in marked decreases of IL-1β secretion from both cell populations (Figure 5.12). Epithelial cells demonstrated a significant reduction in IL-1β secretion when treated with 100nM of MCC950 (76.4 pg/ml of IL-1β secreted compared to 907.8 pg/ml in the LPS and nigericin treated cells; p=0.0357). Treatment with lower concentrations of inhibitor also had a marked effect, although not significant, with 119.3 pg/ml IL-1β secreted and 226.6 pg/ml IL-1β secreted following treatment with 10 nM and 1 nM MCC950 respectively (Figure 5.12 A.). Stromal fibroblasts appeared more sensitive to MCC950 than the epithelial cells which required higher concentrations of the inhibitor in order to achieve a significant reduction in the levels of IL-1β secretion. Stromal fibroblasts secreted 368.7 pg/ml, 50.0 pg/ml and 8.8 pg/ml IL-1β following treatment with 1 nM, 10 nM and 100 nM MCC950 respectively, compared to 3298.3 pg/ml secreted from the un-treated, LPS and nigericin stimulated cells (Figure 5.12 B.).
Figure 5.12: IL-1β production in endometrial cells is dependent on the NLRP3 receptor.

IL-1β secretion from (A.), polarized endometrial epithelial cells and (B.), stromal fibroblasts was examined in the presence of the MCC950 inhibitor, targeting the NLRP3 receptor. Cells were treated with increasing concentrations of the NLRP3 receptor inhibitor MCC950 (1-100 nM) before LPS priming (2 µg/ml) for 6 hours, followed by treatment with nigericin (10 µM) for 1 hour. A DMSO treatment was included as a vehicle control. Supernatants were subsequently harvested and IL-1β levels quantified using a bovine specific IL-1β ELISA (n=5). Statistical analysis was performed using a Mann Whitney test comparing individual inhibitor treatments to the LPS and nigericin stimulation. *=p≤0.05.

Having confirmed the efficacy of the MCC950 inhibitor in bovine endometrial cells, we next needed to examine cell viability following inhibitor treatment, to ensure that the reduced levels of cytokine we observed was due to inhibition of the components required for optimal cytokine release and not due to reduced cell viability or even cell death. Treatment with MCC950 had no effect on epithelial cell viability (as quantified by the measuring trans-epithelial resistance, a measure of epithelial barrier integrity) or stromal fibroblast viability (Figure 5.13 A.-B.).

To ensure specificity of the MCC950 inhibitor to targeting IL-1β, we needed to examine the secretion of other cytokines. Ideally we would have examined secretion of IL-1α, given that it is also a member of the IL-1 family but can be secreted from the cell without NLRP3 inflammasome mediated cleavage. While a previous study reported that bovine endometrial cells were capable of producing low levels of IL-1α (Healy, Cronin et al. 2014), secreted levels of IL-1α by our endometrial cells were below the limits of detection of the ELISA. As endometrial cells have previously been demonstrated to be potent producers of IL-8, we examined the secretion of this cytokine following inhibitor
treatment. MCC950 also had no effect on IL-8 cytokine production by either cell population, indicating its inhibitory effects are specific to IL-1β production (Figure 5.13 C.-D.).

**Figure 5.13:** Cell viability and IL-8 cytokine production are unaffected by treatment with the NLRP3 receptor inhibitor MCC950.

Endometrial cell viability and ability to secrete IL-8 was examined following inhibitor treatment. (A.-B.). The effect of the inhibitor MCC950 on epithelial cell or stromal fibroblast viability was examined by analysing trans-epithelial resistance (TER) or performing a Cell Titer Blue™ viability assay respectively \((n=5)\). (C.-D.). IL-8 cytokine production by epithelial or stromal cells following treatment with MCC950 was measured by ELISA \((n=5)\).

Having confirmed the role of the NLRP3 receptor in inflammasome mediated IL-1β secretion, next needed to examine the role of inflammatory caspases in mediating IL-1β secretion from endometrial cells.
5.3.4 Inflammasome activation in endometrial cells is caspase-dependent

The inflammatory caspases provide another potential target on the inflammasome pathway for IL-1β inhibition, as inflammatory caspases have been implicated in both canonical and non-canonical inflammasome pathways. A wide number of caspase inhibitors have been developed and are commercially available. However, issues with the specificity of inhibitors targeting specific caspases have made it difficult to elucidate the contribution of individual caspase enzymes to IL-1β processing and secretion. In order to circumnavigate this problem, we began by using a pan-caspase inhibitor, allowing us to first determine if IL-1β processing in endometrial cells is caspase dependent before we began elucidating the contribution of individual caspases.

Treatment with increasing concentrations of Z-VAD-FMK resulted in a marked reduction in IL-1β production by both epithelial cells and stromal fibroblasts, with the stromal fibroblasts appearing more sensitive to the inhibitor than the epithelial cells, similar to what was observed with MCC950 treatment (Figure 5.14). In polarized epithelial cells treatment with 100 ng/ml Z-VAD-FMK results in a significant reduction in IL-1β secretion (53.9 pg/ml IL-1β versus 1427.8 pg/ml IL-1β in cells treated with LPS and nigericin alone; p=0.0286) (Figure 5.14 A.). In comparison, treatment of stromal fibroblasts with 1 ng/ml Z-VAD-FMK is sufficient to significantly reduce levels of IL-1β, with levels reduced from 3756.5 pg/ml IL-1β secretion to 188.3 pg/ml (p=0.0044). Treatment with higher concentrations such as 100 ng/ml Z-VAD-FMK completely abrogates IL-1β secretion from stromal fibroblasts (reducing IL-1β secretion levels to 5.2 pg/ml; p=0.0022) (Figure 5.14 B.).
Figure 5.14. IL-1β production in endometrial cells is dependent on caspase activity.

IL-1β secretion from (A.), polarized endometrial epithelial cells and (B.), stromal fibroblasts was examined in the presence of the pan-caspase inhibitor Z-VAD-FMK. Cells were treated with increasing concentrations of the Z-VAD-FMK inhibitor (1-100 ng/ml) before LPS priming (2 µg/ml) for 6 hours, followed by treatment with nigericin (10 µM) for 1 hour. A DMSO treatment was included as a vehicle control. Supernatants were subsequently harvested and IL-1β levels quantified using a bovine specific IL-1β ELISA (n=5). Statistical analysis was performed using a Mann Whitney test comparing individual inhibitor treatments to the LPS and nigericin stimulation. *=p≤0.05.

As detailed previously, with any kind of inhibitor experiment the specificity and off-target effects of these inhibitors, such as reduced cell viability or inhibition of unintended targets, must be examined. Similar to what we had described with MCC950 treatment, the effect of the inhibitor on cell viability and specificity of the inhibitor to inhibiting IL-1β secretion was examined. Treatment of endometrial cells had no effect on endometrial epithelial cell barrier function (indicated by measuring the TER), a surrogate marker for cell viability, or on stromal fibroblast viability (as examined by a cell viability assay) (Figure 5.15 A.-B.). Similarly, the secretion of the chemokine IL-8 by both endometrial cell populations remains unaffected by Z-VAD-FMK treatment (Figure 5.15 C.-D.).
Endometrial cell viability and ability to secrete IL-8 was examined following inhibitor treatment. (A.-B.). The effect of the inhibitor Z-VAD-FMK on epithelial cell or stromal fibroblast viability was examined by analysing trans-epithelial resistance (TER) or performing a Cell Titer Blue™ viability assay respectively (n=5). (C.-D.). IL-8 cytokine production by epithelial or stromal cells following treatment with the pan-caspase inhibitor Z-VAD-FMK was measured by ELISA (n=5).

The use of the pan-caspase inhibitor Z-VAD-FMK has allowed us to confirm that IL-1β secretion from endometrial cells is caspase dependent. However, if we are contemplating the potential therapeutic implications of this finding, and given the role caspase enzymes play in programmed cell death, broadly inhibiting their effects in-vivo may have implications for the processes of uterine involution and tissue remodelling postpartum. Therefore, a more targeted approach to inhibiting caspase activity is needed and in order to achieve this we need to examine the contribution of individual caspase enzymes to IL-1β secretion.
5.3.5 Inflammasome activation in endometrial cells is caspase-4 dependent

Canonical NLRP3 inflammasome pathway activation, as documented in human and murine studies, typically requires caspase-1 for IL-1β cleavage into its pro-form for release from the cell. Previous work in bovine PBMCs has demonstrated caspase-1 expression and identified its role in mediating IL-1β production (Harte, Gorman et al. 2017). However, our previous RNA-seq experiments showed no expression of caspase-1 in our endometrial biopsies whether from healthy or diseased animals, while caspase-13, among a number of other inflammatory caspases, was found to be differentially expressed 21 DPP between healthy and endometritic cows (Foley, Chapwanya et al. 2015).

Caspase-13 has previously been reported as a novel bovine caspase (Koenig, Eckhart et al. 2001). However bioinformatic analysis, aligning the sequence of caspase-13 to caspase-4, has revealed the sequence of capase-13 to be identical to caspase-4 (Figure 5.16). Going forward, we will only refer to caspase-4.

Figure 5.16. Alignment of bovine caspase-4 and caspase-13 reveals 100% homology between the two protein sequences.

Amino acid sequences of bovine capase-4 and bovine caspase-13 were downloaded from GenBank and aligned using Clustal Omega. Amino acids are coloured according to their physiochemical groupings. * indicates identical residues at a position in the alignment.
It has previously reported in the literature that human caspase-4 works in tandem with caspase-5, while in the mouse, caspase-11 is the ortholog of caspase-4 and caspase-5. However, the bovine ortholog has not been examined, and the question over whether bovine caspase-4 is capable of working independently or, if similar to the human, requires the assistance of caspase-5 (or its bovine ortholog) remains unanswered. Similar to the murine genome, caspase-5 not annotated in the bovine. An orthology search was performed using the Ensembl genome browser which found Human caspase-5 mapped to both murine and bovine caspase-4. This confirmed that bovine caspase-4 is the ortholog of caspases-4 and 5 in the human and caspase-11 in the mouse.

In order to investigate the relationships between the different caspase family members, a phylogenetic tree was constructed using the amino acid sequences of the pro-enzyme caspase sequences. Phylogenetic analysis involved using a maximum likelihood model to examine the evolutionary relationships between caspase family members. The phylogeny was tested using a bootstrap test. Bootstrap testing is a resampling analysis tool that operates by taking columns of characters out of analysis and then rebuilds the alignment. This test is to see if nodes are recovered in multiple iterations of the phylogenetic analysis.

Phylogenetic analysis segregates the caspase family into two major subfamilies based on their function, namely the inflammatory caspases and the caspases involved in apoptosis and other forms of programmed cell death (Figure 5.17). The inflammatory caspases are indicated by the red line in Figure 5.17. The inflammatory caspases consist of caspase-1 across human, mouse and bovine; caspase-4 across human and bovine, caspase-11 in mice (the murine ortholog of caspase-4), and human caspase-5. The remaining caspase enzymes have all demonstrated roles in cell death pathways.

In the case of the inflammatory caspases, bovine and human caspases associate more closely than murine caspases. Evolutionarily, human and bovine appear more closely related than mouse. More relevant animal model for inflammatory studies. Both human and bovine caspase-1 and caspase-4 are more closely related than their murine counterparts. This result would indicate that the murine studies may not be as informative when it comes to examining bovine caspase function.

The close evolutionary relationship between caspase-1 and caspase-4 across the three species examined would indicate the potential conservation of function across these
inflammatory caspases. In humans, caspase-1 and caspase-4 have been shown to demonstrate identical substrate specificity. Given the lack of caspase-1 expression in both our previous RNA-seq data and our inability to detect mRNA expression in endometrial cells by qPCR, a potential role for caspase-4 in inflammasome mediated activation of IL-1β in endometrial cells is indicated. We aimed to determine expression of caspase-4 in endometrial cells and elucidate its contribution to inflammasome mediated IL-1β cleavage and release form the cell through the use of caspase-4 specific inhibitors.
Figure 5.17. Phylogenetic analysis of the caspase family indicates the close relationship between caspase-1 and caspase-4.

The evolutionary history of the caspase family across human, mouse and bovine species was inferred using the Maximum Likelihood method based on the Le-Gascuel method (Le and Gascuel 2008). The analysis involved 28 amino acid sequences and was performed using 500 bootstrap replications. Relationships are based on the full length pro-enzyme sequences. All positions with less than 95% site coverage were eliminated (i.e. fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position). The location of the caspase-1 and caspase-4 proteins is indicated by the red bar. Evolutionary analysis was conducted in MEGA X.
Having observed significant differential expression of caspase-4 between healthy and endometritis cows at 21 DPP in our RNA-seq data and identified significant upregulation of caspase-4 mRNA in our endometrial cell populations, we next aimed to detect expression of caspase-4 at the protein level. While no bovine specific caspase antibodies are currently commercially available, we examined a number of human caspase antibodies. Despite the high level of conservation between bovine and human caspase-4, human caspase-4 antibodies were unable to cross-react. The lack of commercially available species-specific antibodies compromises protein expression studies of these inflammatory caspases within the bovine. Additionally, due to the high levels of similarity between caspase-1 and caspase-4 most probes and inhibitors cannot successfully distinguish between the two. Thus caution must be exercised when analysing caspase expression.

The FAM FLICA® flow cytometry based assay has previously been shown to cross react with bovine caspases (Harte, Gorman et al. 2017). However, this assay is limited by its inability to discriminate between caspase-1 and caspase-4, giving a combined report on active caspase-1 and caspase-4 activity. In order to examine caspase expression with this assay, PBMCs, endometrial epithelial cells and stromal fibroblasts were primed with LPS for 3 hours followed by stimulation with the inflammasome activator nigericin for 1 hour. Cells were then treated with the caspase detection probe FLICA 660-YVAD-FMK for 30 minutes. The probe is comprised of an affinity sequence (YVAD) found in both caspase-1 and caspase-4. The probe is cell permeable and binds irreversibly to active caspase-1 or caspase-4 present in cells, emitting a red signal inside cells positive for either active caspase-1 or caspase-4. The presence of this red signal could then be quantified by flow cytometry.

Caspase positive cells were determined following a gating strategy to identify single cell populations of live cells. Cells were initially analysed on a FSC-A vs. FSC-H plot to exclude doublets, followed by FSC-A vs. SSC-A to omit debris. Dead cells were excluded using the 7AAD live/dead stain before gating on FLICA+ cells (Figure 5.18).
Figure 5.18. Caspase-1/caspase-4 activity in endometrial cells determined using the FAM-YVAD-FMK FLICA Assay.

Expression of active levels of caspase-1/caspase-4 was examined. Gating strategies for PBMCs, epithelial cells and stromal fibroblast are shown. Cells were seeded at a density of 0.5×10⁶ and allowed to rest overnight before stimulation with LPS (2 µg/ml) for 3 hours followed by nigericin (10 µM) for 1 hour. Following stimulation PBMCs were re-suspended and endometrial cells lifted and incubated for 30 mins at 37°C with the caspase probe (FAM-YVAD-FMK). Cells were analysed by flow cytometry using a BD Accuri C6 flow cytometer. Cells were analysed on a FSC-A vs. FSC-H plot to exclude doublets followed by FSC-A vs. SSC-A to omit debris. Dead cells were excluded using the 7AAD live/dead stain before gating on FLICA+ cells. Data was analysed using FlowJo software.

Mean fluorescence intensity of the cells staining positive for caspase-1/4 was examined. We observed highest caspase activity levels in stromal fibroblasts and epithelial cells, which is consistent with the high levels of IL-1β production we observed in these cell populations (Figure 5.19). Levels of caspase activity in PBMCs are consistent with previous observations in bovine PBMCs (Harte, Gorman et al. 2017). Caspase activity levels in the control (untreated) samples was highest in stromal fibroblasts (44,840 mean fluorescence units (MFU)) compared to PBMCs (13,919 MFU) and epithelial cells (38,420 MFU). Levels of caspase activity are at their highest in stromal fibroblasts following LPS priming (50,660 MFU). Caspase activity reduces to 45,560 MFU following stimulation with nigericin alone and 39,560 MFU following combined stimulation with LPS and nigericin. In PBMCs, caspase activity is at its highest following treatment with nigericin alone (15,825 MFU) with 15,117 MFU of caspase activity following stimulation with LPS alone and 13,574 MFU following combined LPS and nigericin treatment. Epithelial cells displayed levels of activity close to stromal fibroblasts, with 38,220 MFU of caspase activity following LPS treatment, 38,420 MFU following nigericin treatment and 42,060 MFU following combined treatment with LPS and nigericin.
Figure 5.19. Bovine endometrial cells and PBMCs display differential activity levels of inflammatory caspases.

Expression levels of caspase-1/caspase-4 in PBMCs, epithelial cell and stromal fibroblasts. Expression of active levels of caspase-1 and caspase-4 were examined by flow cytometry using the FAM-YVAD-FMK FLICA assay following stimulation with the inflammasome activators LPS (2 µg/ml) for 3 hours followed by stimulation with nigericin (10 µM) for 1 hour.

Given the difficulties in elucidating caspase-4 expression at the protein level, we aimed to examine its contribution to IL-1β production by inhibiting its activity through the use of a commercially available inhibitor. As mentioned previously, there are difficulties in selecting inhibitors of caspase-1 or caspase-4 which demonstrate specific inhibition of the selected caspase. Many current commercially available inhibitors marketed as being caspase-1 specific target a sequence present in both caspase-1 and caspase-4. In order to obtain a casase-4 specific inhibitor, the target sequences of a number of commercially available caspase-4 inhibitors were aligned to bovine caspase-1 and caspase-4. The target sequence of Z-LEVD-FMK did not align to caspase-1 and so was used to examine caspase-4 dependent IL-1β expression.

Polarized epithelial cells were left un-treated (control) or treated basolaterally with increasing concentrations (0.02-2 µM) of the inhibitor or with the DMSO vehicle control (1% DMSO) for 1 hour prior to LPS priming basolaterally for 6 hours. Following nigericin treatment for 1 hour, supernatants were sampled from the apical compartment and IL-1β levels analysed by ELISA. Similar treatment conditions were applied to stromal fibroblasts in 2D culture.
Stimulation with both inflammasome activating agents LPS and nigericin were required for IL-1β production by both endometrial epithelial cells (1503.7 pg/ml versus 58.2 pg/ml in control samples) and stromal fibroblast populations (3567.9 pg/ml versus 17.4 pg/ml in control samples) (Figure 5.20). Use of increasing concentrations of the commercial caspase-4 inhibitor Z-LEVD-FMK reduced IL-1β levels to levels similar to what was observed in the control samples in the polarized epithelial cells (108.4 pg/ml following treatment with 2 µM Z-LEVD-FMK) (Figure 5.20 A.) and significantly reduced IL-1β production by stromal fibroblasts (37.9 pg/ml following treatment with 2 µM Z-LEVD-FMK) (Figure 5.20 B.).

Figure 5.20. IL-1β production in endometrial cells is dependent on caspase-4.

IL-1β secretion from (A.), polarized endometrial epithelial cells and (B.), stromal fibroblasts was examined in the presence of inhibitors targeting caspase-4. Cells were treated with increasing concentrations of the caspase-4 inhibitor Z-LEVD-FMK (0.02-2 µM) before LPS priming. A DMSO treatment was included as a vehicle control. Supernatants were subsequently harvested and IL-1β levels quantified using a bovine specific IL-1β ELISA (n=5). Statistical analysis was performed using a Mann Whitney test comparing individual inhibitor treatments to the LPS and nigericin stimulation. *=p≤0.05.

Treatment with the Z-LEVD-FMK inhibitor had no effect on trans-epithelial resistance properties, indicating the epithelial barrier was unaffected and so the inhibitor had no effect on cell viability (Figure 5.21 A.). Stromal fibroblast viability was similarly unaffected (Figure 5.21 B.). Inhibitor treatment also had no effect on the production of the chemokine IL-8 by both cell populations, indicating that its effects are specific to inhibiting IL-1β production (Figure 5.21 C.-D.).
Figure 5.21. Cell viability and IL-8 cytokine production are unaffected by treatment with the caspase-4 inhibitor Z-LEVD-FMK.

(A.-B.). The effect of the inhibitor Z-LEVD-FMK on epithelial cell or stromal fibroblast viability was examined by analysing trans-epithelial resistance (TER) or performing a Cell Titer Blue™ viability assay respectively (n=5). (C.-D.). IL-8 cytokine production by epithelial or stromal cells following treatment with Z-LEVD-FMK was measured by ELISA (n=5).

Given the lack of specificity observed with some commercial caspase inhibitors, we wanted to validate our observations with the commercial caspase inhibitor using siRNA designed to specifically knockdown caspase-4 expression. Additionally, we would have liked to confirm the absence of caspase-1 function to endometrial cell mediated IL-1β production through the use of inhibitor experiments. However, with the uncertainty surrounding the specificity of commercially available caspase-1 inhibitors, coupled with the fact we were unable to perform an siRNA knockdown of caspase-1 (we could not detect expression of caspase-1 at the mRNA level and had no antibody to detect protein expression meaning we had no means to validate mRNA knockdown) this was unfeasible.
In order to confirm the role of caspase-4 in endometrial cell mediated IL-1β production, siRNA targeting bovine caspase-4 specifically was designed. The specificity of the siRNA complexes was examined by performing a BLAST search with the designed sequences. In the BLAST programme, the input DNA sequence is searched for against the entire DNA database. The BLAST search indicated that the siRNA complexes shared 100% homology with bovine caspase-4 and did not align with any other caspases.

Treatment of epithelial cells or stromal fibroblasts with caspase-4 siRNA reduced the levels of caspase-4 mRNA present in both cell populations (Figure 5.22). Epithelial cells did not display a strong reduction in caspase-4 mRNA expression following siRNA transfection (0.2 relative expression units in the LPS treated un-transfected cells compared to 0.07 relative expression units in the LPS treated transfected cells) (Figure 5.22 A.). This is likely to be an artefact of their culturing conditions (epithelial cells were cultured on transwell inserts to allow for their polarization). Epithelial cells needed to be polarized in order to allow for maximum IL-1β production, as we have demonstrated previously. Epithelial cells were grown to near confluency (approximately 85-90% confluent) before transfection with the caspase-4 siRNA. Epithelial cells needed to be closer to full confluency as we needed an intact epithelium at time of stimulation. Cells are normally transfected at a lower level of confluency in order to place them in the log phase of growth, as rapidly proliferating cells are more likely to take up the siRNA transfection complex. More confluent cells tend to reside in the lag phase of growth and so do not proliferate as fast, resulting in less siRNA uptake and poorer transfection efficiency. Stromal fibroblasts demonstrated a significant reduction in caspase-4 expression (0.11 relative expression units in the LPS treated un-transfected cells compared to 0.01 in the LPS treated transfected cells; p=0.0286) (Figure 5.22 B.). Stromal fibroblasts were seeded at a lower density and proliferate rapidly and so are better at taking up the siRNA complex. As stromal fibroblasts are the predominant producers of IL-1β within the endometrium, the effect of caspase-4 knockdown will be more striking if it does indeed play a role in mediating IL-1β release from the cell. Additionally, treatment of both cell populations with scrambled siRNA or with the vehicle control alone had no effect on caspase-4 expression levels indicating that the reduction in caspase-4 expression is due to the presence of the siRNA complexes within the cell.
Figure 5.22. siRNA targeting caspase-4 reduces mRNA expression.

Caspase-4 mRNA expression levels in polarized epithelial cells and stromal fibroblasts was examined by qPCR following transfection with siRNA directed against caspase-4, scrambled (non-targeting) siRNA, a vehicle control or left untreated for 48 hours. Following transfection and prior to qPCR analysis cells were stimulated with LPS (2 µg/ml) and nigericin (10 µM) for 6 hours. Cells were then harvested in TRizol. RNA was extracted and first strand cDNA reverse transcribed. qPCR for caspase-4 was performed and gene Cq values were normalised to the reference gene H3F3A. Columns indicate mean relative expression to H3F3A, error bars indicate standard error of the mean (n=5). Statistical analysis was performed using a Mann Whitney test, comparing an un-transfected treatment to its corresponding transfected sample. *=p≤0.05.

This knockdown of caspase-4 completely considerably impacted IL-1β production in both epithelial cell and stromal fibroblasts (Figure 5.23). Despite the modest reduction in caspase-4 mRNA expression following siRNA treatment, IL-1β levels in epithelial cells transfected with siRNA against caspase-4 were reduced to levels observed in control
samples (918.4 pg/ml in un-transfected LPS and nigericin treated samples compared to 80.9 pg/ml in siRNA transfected LPS and nigericin treated samples) (Figure 5.23 A.). In stromal fibroblasts, IL-1β levels were significantly reduced following caspase-4 knockdown, with IL-1β levels of 4437.6 pg/ml in LPS and nigericin stimulated un-transfected cells reduced to 56.5 pg/ml in cells transfected with caspase-4 siRNA (p=0.0159) (Figure 5.23 B.). Treatment with scrambled siRNA and vehicle control alone had no effect on IL-1β expression from both cell populations, confirming the specificity of the caspase-4 siRNA.
Figure 5.23. siRNA targeting caspase-4 confirms IL-1β production in endometrial cells is caspase-4 dependent.

IL-1β production by endometrial epithelial and stromal fibroblasts was measured by ELISA following caspase-4 knockdown. Following treatment with siRNA directed against caspase-4, scrambled (non-targeting) siRNA, a vehicle control or left untreated for 48 hours cell were stimulated with either LPS alone (2 µg/ml), nigericin alone (10 µM) or in combination for 6 hours. Supernatants were subsequently harvested and IL-1β levels quantified by a bovine specific IL-1β ELISA kit (n=5). Columns indicate mean cytokine expression per ml of media, error bars indicate standard error of the mean. *p≤0.05.

Cell viability in either polarized epithelial cells or stromal fibroblasts was unaffected by siRNA transfection and subsequent stimulation (Figure 5.24 A.-B.). No differences in
IL-8 secretion was observed, demonstrating the selective impact of caspase-4 knockdown on IL-1β production (Figure 5.24 C.-D.).

![Graphs showing cell viability and IL-8 cytokine production](image)

**Figure 5.24. Cell viability and IL-8 cytokine production are unaffected by siRNA targeting caspase-4.**

(A.-B.). The effect of treatment with siRNA targeting caspase-4 on epithelial cell or stromal fibroblast viability was examined by analysing trans-epithelial resistance (TER) or performing a Cell Titer Blue™ viability assay respectively \((n=5)\). (C.-D.). IL-8 cytokine production by epithelial or stromal cells following treatment siRNA targeting caspase-4 was measured by ELISA \((n=5)\).

Having determined that IL-1β cleavage and release from the cell is mediated through a non-canonical inflammasome pathway (dependent on caspase-4) in endometrial cells, we next needed to determine if this non-canonical inflammasome activation had any implications for other caspase-1 mediated processes such as gasdermin D cleavage and the initiation of pyroptosis, which has previously been demonstrated to be a requirement for IL-1β release from the cell.
5.3.6 Endometrial cells display reduced levels of pyroptosis in comparison to immune cells upon inflammasome activation

Inflammasome activation and the subsequent release of IL-1β from cells has been linked to pyroptosis, a lytic form of cell death, in both mouse and human models of inflammasome activation (Lamkanfi and Dixit 2012). It is claimed that the process of pyroptosis following inflammasome activation has been proposed as a potential mechanism for IL-1β release from the cell (Hogquist, Nett et al. 1991, Monteleone, Stanley et al. 2018). Caspase-1 has been demonstrated to mediate the cleavage of gasdermin D and initiate the events of pyroptosis in human and mouse (He, Wan et al. 2015). Having observed that inflammasome action in endometrial cells is caspase-4 dependent, we wondered if caspase-4 could mediate the same effects as caspase-1, cleaving gasdermin D and initiating pyroptosis. As we are limited by reagents available for examining gasdermin D activity in the bovine, we instead had to examine surrogate markers for gasdermin D activity, namely cell viability and LDH mediated cytotoxicity as indicators of pyroptotic activity following inflammasome activation by stimulation with LPS and the inflammasome activator nigericin.

Levels of pyroptosis were quantified using an LDH assay which measures the levels of the cytosolic enzyme lactate dehydrogenase (LDH) which is released from dying cells following membrane rupture. Cell viability was examined using a Cell Titer Blue assay. This assay is based on the reduction of resazurin to resorufin by viable cells. This process results in a change in the spectral properties of the reagent which can then be subsequently quantified by measuring the fluorescent signal. Cell viability was further validated using a real time cell analyser which works on electronic sensor technology. Cells are plated onto electronic sensor plates and incubated within the machine in a normal tissue culture incubator. An electric current is passed through the plate. Proliferating cells will impede the flow of electrons to the sensors while dying cells will lift from the bottom of the plate and result in increased electron flow to the sensors. Thus, cell proliferation and death can be analysed in real time with high levels of accuracy. Combining these technologies will allow us to profile the process of inflammasome mediated pyroptosis within endometrial cells following inflammasome activation.

The results indicate that PBMCs incubated with nigericin at increasing time points show a corresponding decrease in the level of cell viability and a corresponding increase in the
level of LDH release, a marker of cell death (Figure 5.25 A. & D.). A decrease in PBMC viability is clearly observed following 1 hour of nigericin treatment (47.3% viability versus 139.0% viability in cells treated with LPS alone for 12 hours). In comparison, both endometrial epithelial cell and stromal fibroblast cell viability levels remain unchanged as does the low level of LDH release from these cells, despite incubating the cells with nigericin for up to 6 hours (Figure 5.25 B.; C.; E. & F.). The effect of nigericin on endometrial cells was further examined over an incubation period of 48 hours using the xCelligence real time cell analyser system. Endometrial cells were plated and allowed to adhere and proliferate for a period of 24 hours before the addition of LPS. Epithelial cells and stromal fibroblasts continued to proliferate and expand in the presence of LPS. Following a period of 24 hours with LPS treatment, nigericin was added. 6-8 hours following nigericin treatment, both epithelial cells and stromal fibroblasts saw a marked reduction in cell viability, as indicated by a reduction in the cell index value (Figure 5.25 G.-H.). The effect on PBMCs could not be examined using this method as cells had to be adherent.
Figure 5.25. Inflammasome mediated pyroptosis is delayed in endometrial cells compared to PBMCs.

(A-C). Cell viability in epithelial cells, stromal fibroblasts and PBMCs was measured using the Cell Titer Blue™ viability assay following a time course stimulation with the inflammasome priming signal LPS and subsequent stimulation with the inflammasome activator nigericin for 1, 3 or 6 hours (PBMC n=1; endometrial cells n=5). (D-F). Pyroptosis was examined in PBMCs, epithelial cells and stromal fibroblasts by measuring LDH activity following a time course stimulation with the inflammasome priming signal LPS and subsequent stimulation with the inflammasome activator nigericin for 1, 3 or 6 hours (n=5). (G-H). Cell dynamics were examined in epithelial cells and stromal fibroblasts using the xCelligence real time cell analyser system in response to treatment with LPS (2μg/ml) and nigericin (10μM) (n=3).
5.4 Discussion

A role for the NLRP3 inflammasome complex in the processing of IL-1β by professional immune cells such as monocytes and macrophages has long been established. In mice, loss of NLRP3 and caspase-1 completely abrogated IL-1β production (Kuida, Lippke et al. 1995, Li, Allen et al. 1995, Li, Willingham et al. 2008). In the human, the contribution of both NLRP3 and caspase-1 to canonical inflammasome mediated IL-1β production has been examined in detail (Kostura, Tocci et al. 1989, Howard, Kostura et al. 1991, Coll, Robertson et al. 2015). Recently, there has also been a growing appreciation and understanding of the role of the inflammasome in mediating IL-1β production in epithelial cell mediated immune responses. The majority of this work has been carried out in human intestinal epithelial cells (Lei-Leston, Murphy et al. 2017). Inflammasome activation in fibroblasts is even less well characterised.

Examining the mechanisms behind inflammasome signalling within the bovine endometrium adds another layer of complexity, with the introduction of potential species-specific differences, in addition to the potential cell- and tissue-specific differences. Given the discrepancies that exist within inflammasome signalling across species, we aimed to examine the conservation, expression and signalling pathways utilised by NLRP3 inflammasome components operating within endometrial cells in an effort to better understand the mechanisms surrounding the regulation of the pro-inflammatory cytokine IL-1β.

Bioinformatics analysis demonstrated the conservation of the genetic components and predicted protein domains of the necessary inflammasome components required for NLRP3 activation across species. The expression of the relevant inflammasome components was later confirmed within endometrial and PBMC cell populations by qPCR. We identified expression of all necessary inflammasome components within epithelial cells and stromal fibroblasts apart from caspase-1. The lack of caspase-1 expression in the bovine endometrium was also confirmed by the absence of this gene from our previous RNA-seq dataset (Foley, Chapwanya et al. 2015). This was the first indication of a potential variance in the endometrial cell inflammasome signalling pathway.

Additionally, treatment of endometrial cells with the MCC950 inhibitor, targeting the NLRP3 inflammasome receptor, completely abrogated IL-1β production, confirming that
IL-1β production in endometrial cells is NLRP3 dependent. We also confirmed that treatment with MCC950 did not result in any off-target effects such as reduced cell viability or cell death or reduced production of other inflammatory cytokines such as IL-8. The specificity of the MCC950 indicates its potential as a therapeutic in the prevention or treatment of postpartum bovine endometritis. MCC950 is predicted to bind directly to NLRP3, affecting its activation. However, its specificity for NLRP3 leaves other inflammasome pathways, such as the NLRC4 and NLRP1, involved in antimicrobial defence, unaffected (Coll, Robertson et al. 2015). Complete depletion of IL-1β is not desirable, as evidenced by previous work with the monoclonal antibody canakinumab which mediated complete inhibition of IL-1β (Lopez-Castejon and Pelegrin 2012, Dinarello and van der Meer 2013). Canakinumab proved unsuccessful as it was shown to increase the risk of infections in humans and mice. It is clear that complete inhibition of IL-1β is not the goal, inhibition needs to be targeted specifically to the site of pathological inflammation.

While treatment of endometrial cells with a pan-caspase inhibitor confirmed the role of inflammatory caspases in mediating IL-1β secretion, the absence of caspase-1 mRNA expression in our RNA-seq dataset and our inability to detect its expression in our endometrial cell cultures, despite observing its expression in bovine PBMCs, indicates the potential for endometrial cells to utilise a non-canonical inflammasome pathway for IL-1β processing. While previous reports have demonstrated bovine specific alterations in the inflammasome signalling pathway, the differences observed here appear to be cell specific, given that IL-1β secretion by bovine PBMCs has been demonstrated to be caspase-1 dependent (Harte, Gorman et al. 2017).

While caspase-1 expression was absent from our RNA-seq dataset, caspase-4 was significantly differentially expressed between healthy and endometritic animals, suggesting it might play a role in pathological inflammation (Foley, Chapwanya et al. 2015). Caspase-4 expression in endometrial epithelial cells and stromal fibroblasts was confirmed by qPCR analysis. Examining caspase-1 expression at the protein level posed a challenge as no commercial antibodies exist for bovine caspase-1 or caspase-4 and, while we tested a number of antibodies designed to detect human caspases, none proved to cross-react with bovine caspases. The FAM FLICA assay was able to detect active caspase-1/caspase-4 activity within our endometrial cells but was unable to discriminate between them. Interestingly, the FLICA assay demonstrated active levels of caspase-1/4
in unstimulated cells. Given our previous observation that bovine cells (both PBMCs and endometrial cells) contain preformed IL-1β and are capable of secreting low levels of IL-1β in response to stimulation with LPS or nigericin alone, and a previous report demonstrating levels of pre-formed IL-1β present in bovine PBMCs (Harte, Gorman et al. 2017), the high levels of active caspase-1/4 in unstimulated cells observed here supports the hypothesis of bovine cells being better primed to respond to inflammatory stimuli than their human or mouse counterparts. It has been previously demonstrated that caspase-1 was active in unstimulated human monocytes but not in macrophages (Netea, Nold-Petry et al. 2009). The authors proposed that the differences in IL-1β processing reflects the distinct and separate functions of these two cell populations in the immune response. Finally, given that we are unable to detect caspase-1 at the mRNA level and the ability of both the commercial caspase-4 inhibitor Z-LEVD-FMK and the caspase-4 specific siRNA to completely abrogate IL-1β production by both epithelial cells and stromal fibroblasts, we propose that caspase-4 mediates IL-1β cleavage into its active form within the endometrium, demonstrating a non-canonical inflammasome pathway operating within endometrial cells.

Reports on caspase-4 directly mediating IL-1β production are rare, with most studies suggesting it is required for detecting cytosolic LPS and subsequently activating the NLRP3 inflammasome complex or that it plays a role in mediating the cleavage of procaspase-1 into its active form (Sollberger, Strittmatter et al. 2012). It is unusual to observe inflammasome activation and IL-1β secretion in the absence of caspase-1 activation. To our knowledge, only one such report exists, demonstrating a role for caspase-4 mediated processing of IL-1β in the absence of caspase-1. This report examined inflammasome activity in human and murine intestinal epithelial cells and found that caspase-4 in humans (or its ortholog caspase-11 in mice) and not caspase-1 were required for inflammasome activity (Knodler, Crowley et al. 2014). Combined with our observations, this data suggests a potential requirement for alternative inflammasome signalling at mucosal surfaces.

Having observed non-canonical inflammasome signalling operating within endometrial cells, we questioned the benefit of having caspase-4 mediate the cleavage of IL-1β in the endometrium as opposed to caspase-1. Investigating the levels of pyroptosis in endometrial cells, we observed that endometrial cells appear much more resistant to cell death than the professional immune cell populations present in PBMCs. We hypothesize
that this may be due to caspase-4 being either less efficient or unable to mediate the cleavage of gasdermin D into its active form and initiate the events of pyroptosis, which has classically been considered to be required for the release of IL-1β from the cell. In mice, caspase-11 has been shown to be crucial for gasdermin D cleavage and the initiation of pyroptosis (Knodler, Crowley et al. 2014), however we are currently limited by the reagents available to fully investigate the relationship between caspase-4 and gasdermin D expression in endometrial cells. It is also important to note that while we do eventually observe a decrease in endometrial cell viability (following approximately 7 hours incubation with nigericin) this may be due in part to the fact that nigericin is a pore-forming toxin and ultimately toxic to cells and so prolonged stimulation will ultimately result in cell death. Pyroptotic cell death following inflammasome activation in endometrial epithelial cells and stromal fibroblasts would be hugely inefficient, given the structural roles epithelial cells and fibroblasts play in the endometrium and the effect this would have on the endometrium’s attempts to repair and return to a state of homeostasis in the postpartum period.

Here we have described for the first time that inflammasome activation within the bovine endometrium is NLRP3 and caspase-4 dependent, identifying a non-canonical inflammasome signalling pathway operating within the bovine endometrium. In comparison to bovine PBMCs which follow a more canonical inflammasome signalling pathway, ultimately resulting in the activation of caspase-1 and processing of IL-1β, endometrial cells appear more resistant to pyroptotic cell death. A comparison of PBMC and endometrial cell inflammasome activation is summarised in Figure 5.26.
Distinct mechanisms of inflammasome activation have been found to operate within (A.) PBMCs and (B.) endometrial cells. Inflammasome activation in bovine PBMCs follows the canonical route of inflammasome activation with the one distinction of caspase-8 playing a role in IL-1β processing. In bovine endometrial cells, caspase-1 is replaced by caspase-4, which processes pro-IL-1β into its active form allowing it to be secreted from the cell. Bovine endometrial cells also exhibit a delayed onset of pyroptosis.
Our understanding of the mechanisms behind the inflammasome signalling pathway operating within the endometrium is crucial to generating new immunotherapeutic targets for the prevention of uterine disease. Currently, the most common treatment for bovine endometritis is the use of intrauterine antibiotics such as cepharin, which has been demonstrated to improve the reproductive performance of cattle (LeBlanc, Duffield et al. 2002, Kasimanickam, Duffield et al. 2005). Other antibiotics have been trialled but issues with the presence of drug residues of these antibiotics in milk, resulting in the milk having to be withdrawn from commercial supply, have resulted in these antibiotics being removed from use. Additionally, with the looming threat of antibiotic resistance, targeted immunotherapies are becoming increasingly popular. Small molecule inhibitors have in the past proven to be more cost effective than biologics and would ultimately allow for preventative treatment for both clinical and sub-clinical forms of disease as opposed to the current antibiotic treatments which are administered following the presentation of clinical symptoms. The development of a small molecule inhibitor, designed to target inflammasome activation in endometrial cells, and prevent the release of IL-1β may prove successful in the treatment of bovine endometritis.

As a ‘healthy’ level of IL-1β production is required in order to mount an appropriate immune response, complete inhibition of the IL-1β cytokine is not a desirable option. Additionally, while NLRP3 is common across inflammasome activation in multiple cell types, a role for caspase-4 in mediating IL-1β production appears unique to cell populations found at mucosal sites like the endometrium. Thus, local inhibition of caspase-4 within the endometrium provides a viable target for the prevention and treatment of the IL-1β mediated pathological inflammation associated with bovine endometritis. Our work here has enhanced our understanding of the regulatory mechanisms surrounding the switch to pathological inflammation in the postpartum endometrium and identified new therapeutic targets for disease prevention.
Chapter 6 General discussion

The uterus is a major immune organ in the body, undergoing major tissue regeneration immediately postpartum and facing exposure to invading and commensal microorganisms. The uterus requires a physiological level of inflammation in order to maintain the microbiome and prevent bacterial invasion and disease. Furthermore, the uterus is extraordinarily dynamic in terms of endocrine control of its function. For the majority of the year, the gravid bovine uterus is under the influence of progesterone, which has well documented immunosuppressive properties (Clemens, Siiteri et al. 1979). The uterus is required to provide a tolerant environment suitable for pregnancy and to allow for the development of a semi-allogenic foetus (Mor, Cardenas et al. 2011). Immediately after birth, the organ undergoes immense tissue remodelling, shifting to a highly inflammatory state within hours (Hansen 2013). These complex uterine functions are performed by the uppermost functional layer of the uterine wall, termed the endometrium, which is highly responsive and immune competent, composed of a single columnar epithelium barrier overlying stromal fibroblast cells. The primary role of the endometrium is to maintain a state of immunological homeostasis, capable of providing a state of tolerance for pregnancy while also responding appropriately to pathogenic threats and mediating mechanisms of tissue repair. It is now becoming clear that dysregulation of this homeostasis can result in uterine disease and has major implications for reproductive and systemic health in the cow.

Bovine uterine disease, in particular endometritis, has consequences for fertility, cow health and milk production, resulting in implications for animal welfare and serious economic repercussions for the international agricultural industry. While inflammation has developed connotations within the literature as being a pathological process, an inflammatory response immediately post-partum is required for dealing with the dysregulation of the microbiome which occurs post-partum and for mediating mechanisms of tissue remodelling and repair during the process of uterine involution, and is to be considered a normal event (Chapwanya, Meade et al. 2012). However, the molecular and cellular events supporting the propagation of this inflammatory response beyond the normal time frame for resolution and the switch to a pathological inflammatory phenotype, evident in uterine diseases remain poorly understood. Emerging data from our laboratory and others now supports the hypothesis that
endometritis is the result of a switch from a physiological to a pathological inflammatory response postpartum. This switch can be attributed to a number of factors including a dysregulation of the uterine microbiome, the presence of PAMPs, MAMPs or inflammatory metabolites, damage to or complete loss of the epithelial barrier, in addition to the underlying immune, metabolic and genetic status of the cow. All risk factors for the development of uterine disease can, in one form or another, result in compromised epithelial cell function, affecting their ability to regulate inflammation and ultimately leading to pathology.

Previous work by our group has found significantly higher levels of mRNA expression of IL-1 family members in cows with cytological (sub-clinical) endometritis compared to healthy cows at both 7 and 21 DPP (Foley, Chapwanya et al. 2015). In this study we have demonstrated that endometrial tissue obtained from cows with endometritis had higher levels of IL-1β protein than healthy cows sampled at the same time-point, confirming our earlier mRNA observation and demonstrating the local production of this inflammatory cytokine within the endometrium. Our laboratory has also recently demonstrated that cows diagnosed with clinical endometritis had significantly higher levels of IL-1β detectable in their vaginal mucus at both 7 and 21 DPP (Adnane, Chapwanya et al. 2017), demonstrating that the elevated IL-1β expression detected in this study is secreted into the lumen of the uterus where it collects in uterine and vaginal mucus, descending the reproductive tract. IL-1β has long been associated with a number of inflammatory and metabolic pathologies including rheumatoid arthritis and type-2 diabetes in the human (Pare, Mailhot et al. 2017), while in the bovine IL-1β expression has been observed in cattle infected with Mycobacterium avium subspecies paratuberculosis, the causative agent of Johne’s disease (Casey, Meade et al. 2015). Similarly, a role for IL-1β has been associated with resistance to Mycobacterium tuberculosis infection and with the pathogenesis of bovine mastitis (Bourigault, Segueni et al. 2013, Xu, Dong et al. 2018). Given our knowledge of IL-1β association with endometritis, and the strong body of evidence in the literature demonstrating a role for IL-1β in inflammatory pathologies, we hypothesized that inflammasome mediated IL-1β production locally within the endometrium is driving the switch to pathological inflammation evident in bovine endometritis. This study aimed to identify the cellular source of IL-1β within the endometrium, identify the mechanisms controlling its release and determine its potential pathological effects within the endometrium.
In order to elucidate the inflammatory pathways operating within the postpartum endometrium, we first had to identify the cell populations involved, examining the relative contributions of the ‘professional’ immune cells versus the innate parenchymal cells that compose the endometrium such as the epithelial cells and stromal fibroblasts. Using IHC to stain for CD45, a marker of haematopoietic immune cells, we were able to identify neutrophils, NK cells and lymphocytes present in the postpartum endometrium. Macrophages were also observed based on their morphology; however interestingly, they did not stain positive for CD45. There has been no previous evidence in the literature reporting a similar phenomenon. CD45 functions as a tyrosine phosphatase receptor involved in regulating cell growth and differentiation and it is possible that CD45 is being cleaved in an effort to restrict macrophage activity in the postpartum endometrium (Trowbridge, Johnson et al. 1992). Further studies are needed to assess the expression of CD45 by bovine endometrial macrophages, particularly in the postpartum period, and elucidate if this absence of CD45 expression is a mechanism aimed at regulating macrophage function or is simply an artefact of the IHC protocol. While currently limited by bovine specific antibodies available, reports are beginning to emerge detailing immune cell populations in the endometrium (Vasudevan, Kamat et al. 2017), with our work identifying immune cell populations in the postpartum endometrium supporting this.

IHC staining localised IL-1β expression to the epithelial cells and stromal fibroblasts, indicating production is predominantly mediated by the epithelial cells and stromal fibroblasts. While our IHC results demonstrated a clear role for these cells in mediating IL-1β expression, further research into bovine endometrial immune responses is hindered by the lack of a commercially available model of these cells. In order to further investigate the activation and regulatory mechanisms surrounding IL-1β production within these cells we required a physiologically relevant model to allow for detailed hypothesis testing. Endometrial cell cultures, including cell lines, tissue explants and isolated primary cell populations have previously been developed but carried with them certain limitations and so their effectiveness as models required further investigation (Fortier, Guilbault et al. 1988, Staggs, Austin et al. 1998, Herath, Fischer et al. 2006, Borges, Healey et al. 2012). Our investigation into the use of the commercially available BEND cell line found it to be an unreliable model of endometrial cells due to the changes in cell morphology over consecutive passages. We found that while explants maintain their
normal tissue architecture in-vitro, the baseline expression of all cytokines examined was significantly higher than primary endometrial cells. This is most likely due to the presence of DAMPs within the tissue, which activate a pro-inflammatory immune response. We concluded that explants are of limited value in examining the induction of immune responses in this study.

The isolation of pure populations of primary endometrial epithelial cells and stromal fibroblasts provided us with a more appropriate model to examine the cell-specific responses and signalling pathways operating in these cells. Primary cells also provide the advantage of allowing us to observe the inter-animal variation and breed-specific differences in immune responses. The isolation and culture of primary bovine endometrial cells has been widely reported (Fortier, Guilbault et al. 1988, Herath, Fischer et al. 2006), however these studies lack sufficient detail for reproducibility. These protocols have been repeated by other groups with varying levels of success, in part due to the difficulty in obtaining pure populations, with most studies reporting on mixed cell populations of epithelial cells and stromal fibroblasts (Swangchan-Uthai, Lavender et al. 2012, Oguejirofor, Cheng et al. 2015). With modifications to previously published protocols, we optimised a technique that allows for the consistent isolation of pure populations of epithelial cells and stromal fibroblasts. Purity was confirmed by the positive staining for their respective cytoskeletal proteins cytokeratin and vimentin and by observable differences in cell morphology. The absence of immune cell contamination was also confirmed by PCR for the immune cell marker PTPRC. Additionally, given the fact epithelial cells possess a 3D structure in-vivo where the tight junctions between cells allows them to polarize and differentially respond to antigen presented at either the apical or basolateral membrane, we established a model of polarized endometrial epithelial cells to allow us to examine the differential response of these cells to the inflammasome activating signals LPS and nigericin presented at either surface.

Efforts to reflect the distinctive 3D architecture of the endometrium in-vitro using more relevant models such as electrospun scaffolds and organoid culturing systems are being developed (MacKintosh, Serino et al. 2015, Boretto, Cox et al. 2017). While we have achieved some success in this regard with polarized epithelial cells, future work must strive to improve on the models of endometrial culture available, taking advantage of these newly developed culturing systems. Ultimately, a model that includes all relevant cell populations and allows for their correct spatial orientation is required. This would
allow us to examine the crosstalk between the epithelium, stromal fibroblasts and tissue resident immune cells. The development of organoids has value in this regard, allowing for the correct spatial interaction of epithelial cells and stromal fibroblasts. The development of endometrial organoids in human and mouse has shown promise (Boretto, Cox et al. 2017); however the technology has not yet been applied to the bovine.

The induction of IL-1β expression in response to the inflammasome activating agents LPS (a microbial PAMP required to induce pro-IL-1β expression and induce expression of the relevant inflammasome components) and nigericin (a microbial toxin required to activate the NLRP3 inflammasome, ultimately mediating cleavage of IL-1β into its active form) was examined in this study. These stimuli were chosen as they have been widely demonstrated in the literature to be potent inflammasome activating agents (Jo, Kim et al. 2016). While pathological levels of LPS have been detected in the postpartum endometrium (Herath, Williams et al. 2007), the presence of the microbial toxin nigericin has not yet been demonstrated. In a study of inflammasome activation in the intestine, the intestinal microbiome was screened and the microbiota metabolites taurine, histamine and spermine were found to activate the NLRP6 inflammasome and induce IL-18 secretion by intestinal epithelial cells (Levy, Thaiss et al. 2015). Performing a similar screen of the postpartum uterine microbiome could identify metabolites capable of activating the NLRP3 inflammasome in endometrial cells. This would provide a more targeted and physiologically relevant approach to endometrial inflammasome activation, in addition to potentially linking dysregulation of the microbiome to pathological inflammasome activation.

Examining cytokine induction, we found that epithelial and stromal cells display divergent cytokine profiles, in particular, IL-1β expression. We observed higher mRNA and protein induction of IL-1β in stromal fibroblasts compared to epithelial cells and PBMCs (considered the classical producers of IL-1β in the literature). This is a novel role for stromal fibroblasts in mediating inflammatory cytokine production in the endometrium, in line with the growing appreciation for stromal fibroblasts in orchestrating local, organ specific immune responses. Maximum IL-1β protein secretion was also dependent on both inflammasome priming and activating signals, providing strong evidence for IL-1β production being inflammasome dependent in endometrial cells.
One potential caveat of this study was the use of diverse breeds as a source of cells for stimulation. As there is increasing awareness that breed-specific differences in immunological function need to be taken into account, we aim to focus on endometrial cells obtained exclusively from the Holstein-Friesian breed for future work, given that this is the dominant breed in dairy production and has a documented increased susceptibility to endometritis (Sheldon, Williams et al. 2008). Macrophages isolated form Holstein Friesian cows have been demonstrated to be more efficient at bacterial killing and produce more IL-1β compared to the Brown Swiss beef breed (Gibson, Woodman et al. 2016). In addition, previous work has identified immune-specific differences between breeds due to TLR gene polymorphisms, likely to impact on overall disease resistance (Jann, Werling et al. 2008).

Aiming to increase the physiological relevance of our epithelial cell model, we utilised a polarized model of epithelial cells grown on transwell inserts to allow for directional stimulation. IL1B mRNA was differentially induced in the polarized epithelial cells and was preferentially induced following basolateral stimulation. This is in contrast to IL8 and IL6 mRNA which was preferentially induced following apical stimulation. Previous work by Healy et al (2015) has also demonstrated that IL-6 is preferentially secreted into the uterine lumen (Healy, Cronin et al. 2015) which supports our finding. Here, we have demonstrated that secretion of IL-1β protein is also found to be preferentially released apically, representing its release into the uterine lumen.

This polarization and directional stimulation of endometrial epithelial cells has allowed us a greater insight into their immune function at mucosal barriers. Epithelial cells demonstrate more restricted expression of IL-1β, possibly due to their requirement to maintain a level of immunological tolerance at the mucosal barrier for homeostasis. The intact epithelium must remain quiescent to the multitude of innocuous antigens encountered on a daily basis and this is exemplified here based on the poor induction of IL1B compared to stromal fibroblasts. We hypothesize that only when the barrier is disrupted and microbes are able to breach the epithelial barrier and stimulate the epithelial cells from their basolateral membrane do we observe peak IL-1β production from these cells.

With the growing appreciation of the interplay between metabolic pathways and the immune system (O'Neill, Kishton et al. 2016), our future work must incorporate
immunometabolic profiling into the examination of optimal IL-1β production by endometrial cell populations. Human and murine studies involving ‘professional’ immune cells such as macrophages and DCs have demonstrated that glycolysis is required for LPS induced IL-1β activation (Krawczyk, Holowka et al. 2010, Masters, Dunne et al. 2010). Some studies are now beginning to address immunometabolic questions in the bovine (Sheldon, Cronin et al. 2018). The interplay between the immune system and metabolism is hugely relevant to the postpartum cow, given that the majority of postpartum cows experience a state of negative energy balance (NEB) in the immediate postpartum period, whereby the energy requirement for milk production exceeds nutrient intake. Additionally, the abundance of glucose has also been found to be reduced in postpartum cows (Sheldon, Cronin et al. 2018). Evidence is now emerging from endometrial explant studies demonstrating that reduced levels of glucose restrict the production of inflammatory cytokines such as IL-6, IL-8 and IL-1β (Turner, Cronin et al. 2016). Future work needs to establish the metabolic status of endometrial epithelial cell and stromal fibroblast populations immediately postpartum, examining the metabolic requirements for inflammasome activation and IL-1β production by these cells. Differences in metabolic pathways functioning in epithelial cells and stromal fibroblasts may account for the observed differences in IL-1β production between the cell populations and may be a contributory factor to the pathological inflammatory switch we observe in endometritic cows. Severe NEB has previously been linked with more severe/prolonged cases of uterine disease and so improving the metabolic status of the cow pre-partum may provide a potential avenue for prophylactic treatment of uterine disease (LeBlanc 2012).

In this study, we have observed a number of anomalies in IL-1β expression in bovine endometrial cells and PBMCs. Levels of pro-IL-1β were observed in unstimulated epithelial cells, stromal fibroblasts and PBMCs, with low levels of cytokine secretion also observed in unstimulated samples. This had previously been observed in bovine PBMCs (Harte, Gorman et al. 2017). This expression of pro-IL-1β in untreated cell has not been observed in human PBMCs. This would indicate that bovine cells are primed to better respond to antigenic challenge and represents an important species-specific difference in IL-1β expression. This finding also emphasises the importance of the inflammasome complex and its ability to regulate IL-1β secretion in the bovine. Inflammasome
regulation is needed to prevent uncontrolled and excessive secretion of IL-1β which could result in pathological levels of inflammation.

Another interesting observation is the identification of a higher molecular weight isoform of IL-1β in epithelial cell lysates. Given our previous observation that IL-1β is preferentially released from the apical membrane of polarized epithelial cells, we hypothesized that this may be due to the addition of a signalling peptide directing the cytokine for apical release. Previous work in both human and mouse monocytes established that IL-1β does not possess a signal peptide and does not follow the conventional route of protein secretion through the ER-Golgi network, however there is no evidence to suggest this is the case in epithelial cells (Rubartelli, Cozzolino et al. 1990). Previous reports had identified that the process of glycosylation had been used for sorting and directing protein secretion to either apical or basolateral membrane (Yamamoto, Awada et al. 2013). While our efforts at elucidating the nature of this IL-1β variant were unsuccessful, future work must focus on isolating this IL-1β variant from epithelial lysates and determining its amino acid sequence and protein structure by mass spectrometry analysis. Ultimately, we aim to synthesise a recombinant protein of this isoform and examine its effects on endometrial cell populations.

Given the association of IL-1β and endometritis pathology, we questioned the mechanism by which IL-1β drives this heightened inflammatory response. Having observed that stromal fibroblasts exhibit the highest levels of IL-1β expression, we hypothesized that they are playing a key role in propagating the pathological inflammatory response. In order to maintain their high level of IL-1β expression they must be continually exposed to antigenic challenge and to achieve this they must remain exposed to the contents of the uterine lumen, meaning the epithelial barrier must remain disrupted. In this study, we observed that treatment with increasing concentrations of IL-1β results in a delay in the re-establishment of the epithelial barrier, uncovering a potential mechanism by which IL-1β mediates its pathological effects for the first time in cattle. The identification of this link to pathology highlights IL-1β as a potential target for therapeutics aiming to resolve the pathological inflammation associated with endometritis. Future work will clarify whether it is the stromal fibroblast mediated IL-1β production that disrupts the epithelial barrier or prevents its re-establishment in the postpartum period. To better address this, we would need to further refine our experimental approach. Co-culturing stromal fibroblasts with polarized epithelial cells overlying them would allow us to stimulate the
stromal fibroblasts to produce IL-1β prior to the calcium switch assay and allow us to directly examine if the stromal fibroblast secreted IL-1β is affecting the epithelial barrier. Treatment of stromal fibroblasts with specific inflammasome inhibitors such as MCC950 would also allow us to determine if IL-1β inhibition can promote epithelial barrier establishment.

Within the intestine, disruption of the epithelial barrier has been found to contribute to intestinal inflammation in inflammatory pathologies such as Crohn’s disease (Ma 1997). Reports examining the effect of IL-1β on intestinal epithelial cells found that IL-1β was able to directly target specific tight junction proteins such as occludin (Al-Sadi, Ye et al. 2008). Additionally, the interplay between IL-1β and calcium signalling pathways may explain how IL-1β is mediating its pathological effects on the epithelial barrier. Calcium levels play a key role in maintaining epithelial barrier integrity, as exemplified in our calcium switch assay, whereby depleting calcium levels in the cell culture media induced epithelial barrier dysregulation. Our previous RNA-seq study had identified that calcium signalling was elevated in healthy cows at 21 DPP compared to endometritic cows (Foley, Chapwanya et al. 2015), indicating the role of calcium signalling in the resolution of inflammation. This data also demonstrated elevated expression of the S100 proteins S100A8 and S100A9 at 21 DPP in endometritic cows. IL-1β has been demonstrated to induce S100A8 expression in murine fibroblasts (Rahimi, Hsu et al. 2005). S100 proteins have previously been demonstrated as being capable of chelating calcium (Corbin, Seeley et al. 2008). Their induction by IL-1β in the postpartum endometrium may provide a reason for the reduced levels of calcium signalling observed in endometritic cows. In addition, emerging evidence from our group indicates that endometritic cows possess significantly lower circulating vitamin D (1,25(OH)₂D₃) levels (A. Brewer; pers. comm.). Vitamin D is required for calcium absorption and deficiency in vitamin D has previously been demonstrated to result in disruption of the epithelial barrier (Assa, Vong et al. 2014). Levels of vitamin D and calcium are clearly an important requirement for resolution of inflammation in the postpartum endometrium. A role for IL-1β in inducing dysfunction in these signalling pathways provides an interesting avenue for future investigation.

Having observed species-specific differences in IL-1β expression, it was evident that observations reported in human and murine studies may not necessarily translate to the bovine. This had potential implications for the inflammasome signalling complex, where cell and tissue specific differences in the signalling pathway used in human and murine
studies had previously been identified. We therefore examined the conservation, expression and signalling pathway utilised by the inflammasome complex within bovine endometrial cells in order to better understand the inflammatory mechanisms operating in the postpartum endometrium.

Bioinformatic analysis confirmed the conservation of inflammasome components within the bovine genome and indicates a high level of protein conservation. The required signalling regions were present across mouse, human and bovine species. Examining the mRNA expression of individual inflammasome components, we observed expression of all necessary inflammasome components with the exception of caspase-1. This represents the first study to demonstrate the expression of NLRP3 inflammasome components within the bovine endometrium. Through the use of small molecule inhibitors such as MCC950 which targets the NLRP3 receptor, we were able to demonstrate that IL-1β production in endometrial cells is NLRP3 dependent. Through the use of a commercial inhibitor and siRNA specifically targeting caspase-4, we identified that caspase-4 functions in the absence of caspase-1 in bovine endometrial cells, similar to a process previously observed in the intestinal epithelium (Knodler, Crowley et al. 2014). Here, we have identified a non-canonical inflammasome pathway operating within the bovine endometrium for the first time.

The identification of this non-canonical inflammasome pathway operating in bovine endometrial cells holds potential implications for the development of immunotherapeutics. Inflammasome activation utilising caspase-4 in endometrial epithelial cells and stromal fibroblasts allows for targeted inhibition of caspase-4 in these cells while allowing the ‘professional’ immune cell populations, which utilise caspase-1, to continue producing physiological levels of IL-1β. This selective inhibition of IL-1β is preferable as it would not render the cow vulnerable to opportunistic infections. The success of these inhibitors in our in-vitro model indicates their potential role in prophylactic treatment of bovine endometritis. To get this to a stage where it can be trialled in an in-vivo model, a number of aspects require further investigation, including its potential in-vivo ability to regulate inflammasome activation, establishing the in-vivo dose needed, potential toxicity to the cow and if meat and dairy products from treated cows need to be excluded from the food-chain. The route of administration would also need to be investigated, whether to administer systemically by injection into the blood or locally using a uterine wash.
The identification of this non-canonical inflammasome pathway has a number of implications for processes such as pyroptosis which have previously been associated with caspase-1 activation. Pyroptosis following inflammasome activation would be extremely detrimental in the postpartum endometrium, which is trying to repair and restore the tissue to a state of homeostasis following pregnancy and the events of parturition. The continued loss of the epithelium due to caspase-1 activation would result in near constant activation of the stromal fibroblasts and an overwhelming inflammatory response. The utilisation of this non-canonical inflammasome pathway by the endometrial cells provides another example of how the inflammasome complex attempts to regulate and maintain a homeostatic level of inflammation in the endometrium. This is exemplified in a mouse model of colitis where complete knockout of caspase-11 (the murine ortholog of caspase-4) resulted in reduced intestinal epithelial cell proliferation and increased cell death (Oficjalska, Raverdeau et al. 2015).

This study has created a better understanding of the mechanisms surrounding the switch from physiological to pathological inflammation which occurs in endometritic cows postpartum. In particular, we now have a greater understanding of the role of the inflammasome and associated IL-1β production within the endometrium postpartum (summarised in Figure 6.1). We have also identified a number of cell and bovine species-specific differences in inflammatory signalling. We propose that during an early physiological inflammatory response occurring as early as 7DPP, inflammasome activation occurs in response to bacterial stimulation within the newly restored and intact epithelium, with IL-1β preferentially secreted into the uterine lumen where it can activate neutrophils and stimulate HDP production by epithelial cells, initiating an ‘extra-corporeal’ immune response. This inflammatory response is a result of normal tissue repair and involution processes. Stromal fibroblasts are also involved in this process but their activation and response is limited by the presence of an intact epithelium. However, the loss of the epithelial barrier and exposure of the underlying stromal fibroblasts to antigenic stimulation results in inflammasome activation in the persistently exposed stromal fibroblasts and ultimately a switch to a pathological inflammatory response occurring around 21 DPP. The destruction of the epithelial barrier contributes to the presence of DAMPs while the dysregulation of the microbiome ensures there is an abundance of PAMPs. The exposure and activation of the stromal fibroblasts results in an excessive production of IL-1β. This IL-1β is not directionally secreted into the lumen.
and so can remain in the endometrial tissue propagating the pathological inflammatory response, while also directly affecting the epithelium by preventing the re-establishment of the epithelial barrier and ensuring their continued stimulation from luminal antigens. The distinguishing feature between the physiological and pathological inflammatory phenotypes is its regulation. Further work is needed to investigate the contribution of bacterial species present in the uterus postpartum, the level of tissue damage in the postpartum uterus and their contribution to the events surrounding the resolution of the postpartum uterine inflammatory response.
Figure 6.1. Model of the switch from physiological to pathological inflammation in the postpartum bovine endometrium.

During periods of homeostasis, the intact epithelium maintains a state of physiological inflammation, tolerating the presence of commensal bacteria and preventing the underlying stroma from coming into contact with MAMPs while also mediating cytokine production in response to pathogenic challenge. The process of parturition and the resulting mechanism of tissue repair and involution results in damage to epithelial tight junctions or complete loss of the epithelium. Coupled with this is the dysregulation of the uterine microbiome and the release of tissue DAMPs by dying or necrotic tissue. The exposure of stromal fibroblasts to inflammatory stimulants, results in inflammasome activation and IL-1β secretion by these cells and represents a switch to a pathological inflammatory immune response.

Given the rise in global populations and the resulting demand on food production, there is an increasing need to maximise milk production and fertility parameters. Understanding the mechanisms driving inflammatory diseases such as endometritis will contribute to resolution of these issues (Meade 2015). Here we have demonstrated a role for IL-1β in endometrial cell mediated inflammation and have established that its production is regulated through the NLRP3 inflammasome complex. The Scottish surgeon John Hunter wrote in 1794 that “Inflammation in itself is not to be considered a disease” (Majno 1975), and this is particularly relevant when examining the role of IL-1β in the post-partum endometrium. Inflammation within the postpartum endometrium has previously been shown to be essential for restoring homeostasis in an organ that has undergone significant trauma following the process of parturition and the subsequent dysregulation of the microbiome and the process of tissue involution (Chapwanya, Meade et al. 2012). At physiological levels, IL-1β plays a role in returning the organ to a state of homeostasis, driving the inflammatory response that is crucial for pathogen clearance and the destruction and removal of unwanted tissue to allow the uterus return to size. It is the level at which IL-1β becomes pathological, driving a prolonged inflammatory phenotype resulting in clinical manifestations and implications for future fertility and milk yields that has previously been poorly understood. Our work here has opened a window on the complex cellular and molecular regulation of the switch which occurs in the postpartum endometrium from a healthy inflammatory phenotype to a pathological one and will ultimately form the basis for targeted treatment and improved outcomes for bovine endometritis.
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Chapter 8 Appendices

8.1 Solutions

D-PBS (10X)
Dissolved in 800ml H₂O: 80g NaCl, 2g KCl, 14.4g Na₂HPO₄, 2.4g KH₂PO₄, pH to 7.4 and make up to 1000ml.

Carbonate/bicarbonate buffer
Sodium carbonate (0.2M): 2.2g sodium carbonate in 100ml dH₂O.
Sodium bicarbonate (0.2M): 1.68g sodium bicarbonate in 100ml dH₂O.
Combine 4ml of carbonate solution with 46 ml of bicarbonate solution. Add 200ml dH₂O (pH 9.2)

ELISA Blocking buffer
BSA (4g) and 5g sucrose dissolved in 100ml PBS. 0.2µm sterile filtered.

Reagent diluent
BSA (1g) dissolved in 25ml PBS. 0.2µm sterile filtered.

PBS-Tween
Tween-20 (500µl) added to 1000ml PBS.

4% Fish Skin Gelatin (FSG) blocking buffer
4ml Fish Skin Gelatin added to 100ml PBS

RIPA buffer
50mL Tris-HCl (pH 7.4), 30ml 5M NaCl, 10ml Igepal (NP-40), 50ml 10% sodium deoxycholate, 10 mL 10% SDS, 850 mL dH₂O. pH to 7.4.
Loading buffer
4X Tris-glycine SDS β-mercaptoethanol.

Running buffer (10X)
Dissolved in 1L dH₂O: 0.5mM MES (47.6g), 500mM Tris (60.6g), 10mM EDTA (2.92g) and 1% SDS (10g). Dilute to 1X running buffer with dH₂O. Reducing agent must be added fresh to 1X running buffer (0.94g Na₂S₂O₅ added to 1L 1X running buffer).

Transfer buffer (10X)
250mM Tris (30.3g) and 1.9M glycine (140.28g) dissolved in 800ml dH₂O. Final volume adjusted to 1000ml with dH₂O. For 1X transfer buffer 100ml 10X transfer buffer was added to 700ml dH₂O and 200ml methanol.

15% resolving gel
7.5ml acrylamide, 5.6ml 1.5M Tris, 4.2ml dH₂O, 150µl 10% SDS, 150µl APS, 15µl Temed.

4% stacking gel
1.0ml acrylamide, 750µl 1.5M Tris, 4.61ml dH₂O, 60µl 10% SDS, 60µl APS, 12µl Temed.

Antigen retrieval buffer
Solution A: 0.1M Citric Acid (21.014g in 1L dH₂O).
Solution B: 0.1M Tri-sodium citrate (29.410g in 1L dH₂O).
To prepare antigen retrieval buffer combine 26ml solution A with 124ml solution B and 1,350ml dH₂O. Adjust pH to 6.

FACS buffer
PBS containing 5% FBS and 0.1% sodium azide.
8.2 Primers used for gene expression analysis

Primer details are recorded in (Table 8.1). Lyophilised primers were re-suspended to a working concentration of 50µM. Primer mixes were prepared by adding 25µl of forward and reverse primer to 200µl dH₂O to achieve a final concentration of 5µM for each primer.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Size</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1A</td>
<td>TAGCTCGGTTCAAGAAAGAAGT</td>
<td>GTCTCCACGGAAGTGCTCAT</td>
<td>164bp</td>
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<td>Gene</td>
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<td>Reverse Primer</td>
<td>Length</td>
<td>Concentration</td>
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