REPRODUCTION
REVIEW

Innate immunity and inflammation of the bovine female reproductive tract in health and disease

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Abstract

Mammalian reproductive physiology and the development of viviparity co-evolved with inflammation and immunity over millennia. Many inflammatory mediators contribute to paracrine and endocrine signalling, and the maintenance of tissue homeostasis in the female reproductive tract. However, inflammation is also a feature of microbial infections of the reproductive tract. Bacteria and viruses commonly cause endometritis, perturb ovarian follicle development and suppress the endocrine activity of the hypothalamus and pituitary in cattle. Innate immunity is an evolutionary ancient system that orchestrates host cell inflammatory responses aimed at eliminating pathogens and repairing damaged tissue. Pattern recognition receptors on host cells bind pathogen-associated molecular patterns and damage-associated molecular patterns, leading to the activation of intracellular MAPK and NFκB signalling pathways and the release of inflammatory mediators. Inflammatory mediators typically include the interleukin cytokines IL1β and IL6, chemokines such as IL8, interferons and prostaglandins. This review outlines the mechanisms of inflammation and innate immunity in the bovine female reproductive tract during health and disease condition.

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Introduction

Inflammation is a cellular reaction triggered by noxious stimuli, including infection and tissue injury, which evolved as an adaptive response for restoring homeostasis in the cells and tissue of simple organisms (Medzhitov 2008). Before the appearance of receptors and signalling cascades, cytokines evolved as intracellular messengers in invertebrates, where they play a role in host defence and repair (Dinarello 2007). The general term 'cytokine' includes several families of proteins including interleukins (IL), chemokines, interferons, mesenchymal growth factors, adipokines and tumour necrosis factors (TNF), whilst typical lipid mediators of inflammation include prostaglandins and resolvins. Most of these inflammatory mediators are soluble factors that are produced by one cell and act on another target cell to change its function; some consider these mediators as the 'hormones' of the immune and inflammatory responses (Dinarello 2007). The systems of inflammation and innate immunity have co-evolved with reproductive physiology, and the development of viviparity over 340 million years. Several inflammatory mediators have been co-opted and used for signalling processes during evolution of the reproductive system, and have primary roles in physiology rather than inflammation. Inflammation is also part of maintaining tissue homeostasis during physiological changes in architecture, such as ovulation or parturition. However, innate immunity remains vital to protect the female reproductive tract from the adverse effects of microbial infections. These infections are particularly important in *Bos taurus*, where bacteria and viruses commonly cause infertility. This review outlines the mechanisms of inflammation and innate immunity in the bovine female reproductive tract during health and disease.

Inflammation

After infection or tissue injury, inflammatory mediators are released resulting in the cardinal signs of

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inflammation: redness, heat, swelling, pain and loss of function. Inflammatory mediators typically include cytokines such as IL1β, IL6 and TNFα; chemokines such as IL8 and prostaglandin E_2. These mediators initially direct the inflammatory response by attracting and activating immune cells, particularly neutrophils and macrophages, to remove microbes and damaged host cells. Later, inflammatory mediators such as IL10 and resolvins foster the repair of tissue and coordinate the timely resolution of inflammation. However, multiple cytokines are involved in the inflammatory response, many have pleiotropic effects, and they often duplicate the function of each other.

Innate immunity

Innate immunity encompasses several facets of non-specific protection against microbial infection and tissue damage in the female reproductive tract. The obvious anatomical barriers comprising the vulva, vagina and cervix counter ascending microbial infections reaching the uterus. Other physical barriers in the genital tract include the stratified squamous epithelium of the vagina, the columnar epithelium of the endometrium, the basement membrane of ovarian follicles and the zona pellucida of the oocyte. Antimicrobial peptides and mucosal glycoproteins also cover the mucosa to neutralise bacteria and prevent them reaching the epithelium. The principal cysteine-rich, cationic, antimicrobial peptides expressed in the bovine endometrium include β-defensins, lingual antimicrobial peptide (LAP), and tracheal antimicrobial peptide (TAP), and their transcripts are more abundant in the face of microbial challenge (Davies *et al.* 2008, Chapwanya *et al.* 2013). Other surface molecules that may help to protect the endometrium include the mucins; evidence for their role includes genetic deletion of *Muc1* in mice, which is associated with chronic inflammation of the lower female reproductive tract by opportunistic bacterial infections (DeSouza *et al.* 1999). The expression of mRNA encoding acute-phase proteins in the uterus and ovary is also of interest, because they may provide further localised protection (Fischer *et al.* 2010, Lecchi *et al.* 2012, Chapwanya *et al.* 2013). Although, it is possible that the more usual hepatic production of acute-phase proteins in response to increased serum concentrations of IL6 may be more relevant *in vivo*. The complement system is also present in the female reproductive tract, and this series of related proteins opsonise infected cells to attract immunoglobulin and drive the formation of the membrane attack complex leading to cytolysis. However, normal cells in the reproductive tract are protected against formation of the complement complex by complement regulatory proteins including CD46, CD55 and CD59 (Jensen *et al.* 1995).

Whilst physical barriers and secreted proteins are important for passive defence against microbes in the female reproductive tract, there are some caveats. First, microbes have evolved strategies to avoid many host defences. For example, some bacteria produce enzymes that digest mucus to penetrate the protective layer, and many bacteria secrete proteases that can disrupt protective peptides and proteins produced by the host (Baxt *et al.* 2013). Second, the rapid inflammatory responses associated with microbial infections are associated with pattern recognition receptors of the innate immune system, rather than passive defence systems. Finally, there is also a role for adaptive immunity in the bovine reproductive tract, with the presence of plasma cells in the endometrium and accumulation of immunoglobulins (Anderson *et al.* 1996). However, the mechanisms of adaptive immunity in the female reproductive tract are not part of the present review.

Innate immunity is predicated on host cells proteins, called pattern recognition receptors that are able to sense pathogens or danger. Innate immunity is evolutionarily conserved across animal phyla, and the first pattern recognition receptor to be identified was Toll in *Drosophila melanogaster* (Lemaitre *et al.* 1996). Toll-like receptors (TLRs) and Nod-like receptors (NLRs) are the two most studied families of pattern recognition receptors in mammals, and some of their ligands are listed in Table 1. The TLRs and NLRs principally bind pathogen-associated molecular patterns (PAMPs) found in prokaryotes, but not eukaryotes (Beutler 2009, Takeuchi & Akira 2010). However, danger signals and endogenous damage associated molecular patterns

Table 1 Pattern recognition receptors, their localisation and the pathogen-associated molecular patterns they bind.

Receptor	Localisation	Pathogen-associated molecular patterns
TLR1	Plasma membrane	Triacylated bacterial lipopeptides
TLR2	Plasma membrane	Bacterial lipopeptides, glycolipids, peptidoglycan
TLR3	Endosome	Viral double-stranded RNA
TLR4	Plasma membrane/ endosome	Lipopolysaccharide
TLR5	Plasma membrane	Flagellin
TLR6	Plasma membrane	Diacylated bacterial lipopeptides
TLR7	Endosome	Viral and bacterial single-stranded RNA
TLR8	Endosome	Viral and bacterial single-stranded RNA
TLR9	Endosome	CpG-rich bacterial and viral DNA
TLR10	Plasma membrane	Unknown
RIG-I	Cytoplasm	Short double-stranded RNA
NOD1	Cytoplasm	D-glutamyl-meso-diaminopimelic acid
NOD2	Cytoplasm	Muramyl dipeptide
NLRP3	Cytoplasm	Uric acid crystals, cholesterol-dependent cytolysins
NLRC4	Cytoplasm	Flagellin, type III secretion system proteins

(DAMPs), such as high-mobility group box 1 protein (HMGB1), ATP, nucleic acids and IL1α, released from damaged or dead cells, are also thought to bind pattern recognition receptors to initiate an inflammatory response (Chen & Nunez 2010). The most important TLRs for the recognition of bacteria are TLR1, TLR2 and TLR6, which form heterodimers to bind bacterial lipopeptides (Fig. 1), and TLR4, which binds the cell wall component of Gram-negative bacteria lipopolysaccharide (LPS, endotoxin), in complex with CD14 and MD2 (Takeuchi & Akira 2010). The NLRs, such as NODs and NLRP3, are intra-cytoplasmic receptors that recognise endogenous molecules as well as microbial molecules. Binding of PAMPs to pattern recognition receptors stimulates the production of inflammatory mediators, which recruit neutrophils and monocytes to the site of infection (Beutler 2009, Takeuchi & Akira 2010). In addition, NLR activation leads to the formation of the multi-protein inflammasome complex, characteristically leading to cleavage of caspase-1 to an active form and production of mature IL1β (Schroder & Tschopp 2010).

The innate immune response to PAMPs and DAMPs is most commonly driven by macrophages, neutrophils and dendritic cells. However, a range of cells other than 'professional' immune cells also express pattern recognition receptors. For example, endometrial epithelial and stromal cells express the TLR4–CD14–MD2 receptor complex necessary to detect LPS, and respond to LPS by secreting IL6, IL8 and prostaglandin E_2 (Herath *et al.* 2009*a*, Cronin *et al.* 2012). So, the overall inflammatory response to pathogens associated with innate immunity depends on the sum of the actions of multiple cell types rather than just specialised immune cells. Furthermore, host cellular responses to pathogens are greater than when the same cells encounter commensal microbes. The impact of pathogens on the host is enhanced by a multitude of virulence factors including pore-forming toxins, bacterial secretion systems and bacterial proteases (Blander & Sander 2012). In addition, unlike commensal organisms, pathogens not only colonise tissues but also invade the cells of the female reproductive tract, often causing cell death (Donofrio *et al.* 2007, Sheldon *et al.* 2010). Therefore, the scale of the inflammatory response to microbes is dependent on multiple factors including microbial virulence effectors, host tissue factors, and on the regulation of intracellular signalling pathways associated with innate immunity.

The adaptor molecule MYD88 is essential for downstream signalling of all TLRs, with the exception of TLR3, although additional adaptor proteins are also involved with some of the TLR dimers (Fig. 1; Takeuchi & Akira 2010, Moresco *et al.* 2011). Once activated, MYD88 stimulates IL1R-associated kinases, which in turn activate TNFR-associated factor 6 (TRAF6) that catalyses a complex consisting of TGFβ-activated kinase 1 (TAK1) and the members of the TAK1-binding protein (TAB) family. This complex activates two important pathways in inflammation: the MAP kinase pathway and the NFκB pathway. The MAP kinase cascade (JNK, ERK1/2 and p38) activates the transcription factor AP1, which along with NFκB drives transcription in the nucleus (Fig. 1).

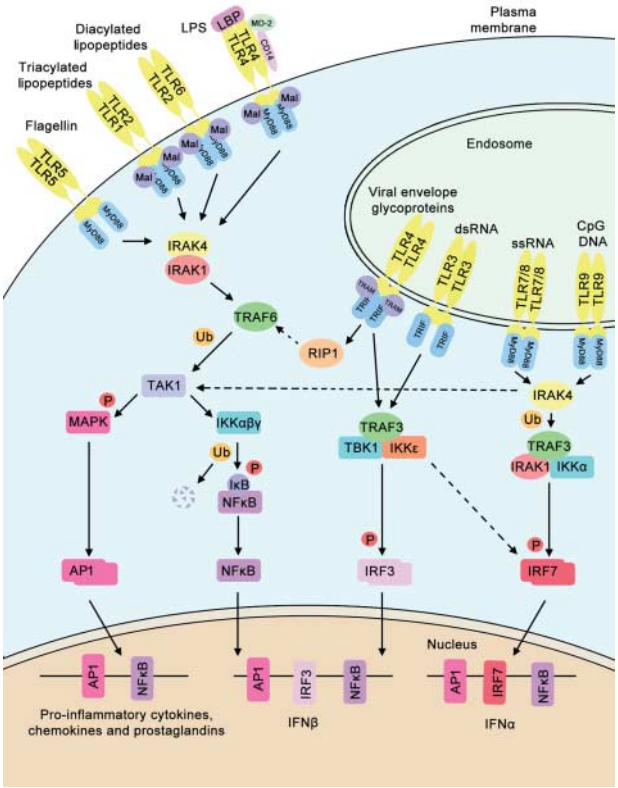

Figure 1 Signalling pathways of the Toll-like receptors. During infection, PAMPs initiate signalling pathways by interacting with TLRs, resulting in formation of homo- or heterodimers and activation of cytosolic Toll/IL1 receptor (TIR) domains. Downstream signalling pathways are initiated through four adaptor proteins: MYD88, MAL, TRIF or TRAM. All TLRs, with the exception of TLR3, use the MYD88-dependent pathway. MAL acts as a bridge to recruit MYD88 to TLR2 and TLR4 signalling. The adaptor TRIF is used in TLR3 signalling and, in association with TRAM, in endosomal TLR4 signalling. In the MYD88-dependent pathway, MYD88 associates with IRAK4, IRAK1 (and/or IRAK2; not illustrated). IRAK4 in turn phosphorylates IRAK1 and promotes association with TRAF6, which serves as a platform to recruit and activate the kinase TAK1. Activated TAK1 leads to the activation of the MAPK cascade. The MAPKs ERK, JNK and P38 enhance the expression of pro-inflammatory cytokines via activation of AP1 (for simplicity, only activation of AP1 is illustrated in the diagram). TAK1 also leads to the activation of the IKK complex, composed of IKKα, IKKβ and IKKγ, which results in the phosphorylation and ubiquitination of IκB. Ubiquitination results in the degradation of IκB, freeing NFκB to translocate from the cytoplasm to the nucleus, where it activates gene expression in concert with AP1. Endosomal TLR3 and TLR4 signalling, through the adaptor protein TRIF, leads to the activation of TBK1 or IKKε, inducing IRF3 dimerisation and translocation into the nucleus where it induces transcription of type I IFN and IFN-inducible genes, including IFNβ. Downstream of TLR7; TLR8; and TLR9, IRF7 is directly phosphorylated by IRAK1 and translocates into the nucleus to induce the expression of type I IFN and IFN-inducible genes, including IFNα.

Typical genes transcribed by activation of these MAPK and NFκB signalling pathways from surface TLRs include cytokines (*IL1A*, *IL1B*, *IL6* and *TNFA*), chemokines (*CXCL8* and *CXCL1*), and lipid mediators (*PLA2*, *PGHS* and *PGES*). On the other hand, internal TLRs activate signalling pathways in response to microbial nucleotides that lead to the expression of type I interferons (Fig. 1).

Inflammation and reproductive physiology

There are extensive reviews about the role of inflammatory mediators and inflammation in reproductive physiology (Richards *et al*. 2002, 2008, Jabbour *et al*. 2009, Turner *et al*. 2012). In particular, physiological events within the ovary, including ovulation and corpus luteum formation and regression, are inflammatory-like events (Espey 1980). Whilst the luteinising hormone (LH) surge is the main trigger for ovulation, inflammatory mediators regulate several of the events around the time of ovulation. For example, IL6 regulates ovarian cumulus granulosa cell function, cumulus–oocyte complex expansion, and oocyte competence, at least in mice (Liu *et al*. 2009). The process of ovulation involves rupture of the dominant follicle, which causes tissue damage, and so it is not surprising that innate immunity and inflammation have roles in the maintenance of tissue homeostasis in the ovary (Richards *et al*. 2008). In the mouse, TLR4 may have a role in normal cumulus–oocyte complex expansion and ovulation by binding endogenous ligands such as hyaluronic acid (Shimada *et al*. 2008). After ovulation, the oocyte enters the uterine tube (oviduct), and although many neutrophils are present, prostaglandin E_2 released by uterine tube epithelial cells after LH stimulation suppresses the phagocytosis of sperm by neutrophils, thereby supporting sperm survival in the uterine tube (Marey *et al*. 2014). As well as responding to LH, the uterine tube epithelial cells have innate immune capabilities, expressing TLR2 and TLR4, and generating inflammatory responses to LPS (Kowsar *et al*. 2013). The LH surge also stimulates localised production of IL8 and angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor A, which are essential for the development of the corpus luteum (Schams *et al*. 2001, Berisha *et al*. 2006, Jiemtaweeboon *et al*. 2011). Lymphangiogenesis is also important in the bovine corpus luteum, with lymphangiogenic factors expression particularly in the corpus luteum of mid-cycle as well as early pregnancy (Nitta *et al*. 2011). If cattle are pregnant, then interferon tau released by the trophoblast prevents the development of the luteolytic mechanism by inhibiting the expression of the oxytocin receptor gene in the endometrial epithelium, which prevents the release of luteolytic pulses of prostaglandin $F_{2\alpha}$ (Dorniak *et al*. 2013). Whereas, in the absence of an embryo, prostaglandin $F_{2\alpha}$ induces luteolysis with the assistance of other inflammatory mediators such as TNFα (Skarzynski & Okuda 2010).

Parturition and the *postpartum* period

Progesterone maintains uterine quiescence throughout gestation, but before parturition the influence of progesterone diminishes rapidly. In several species, the reduction in the influence of progesterone is associated with an increase in inflammatory mediators, such as IL1β, IL6, IL8, and TNFα, and an influx of leukocytes (Challis *et al*. 2009). Similar mechanisms are likely important in cattle, as there is increased expression of genes such as *CXCL8, CXCL2* and *CXCL6* in the placentomes of healthy cattle during parturition (Streyl *et al*. 2012). Another feature of the peripartal bovine uterus is the accumulation of $CD14^+$ monocytes and macrophages in the caruncular stroma and septa, although not in the foetal part of the placentomes (Miyoshi & Sawamukai 2004, Oliveira & Hansen 2009). Activated macrophages enhance the local inflammatory response by attracting neutrophils and monocytes, which clear apoptotic cells, and remodel the caruncle, aiding the successful release of the foetal membranes. Whilst the distribution and concentration of $CD14^+$ cells is similar in healthy cows and cows that develop retained foetal membranes, the latter have lower concentrations of acid phosphatases, a lysosomal enzyme of macrophages (Miyoshi *et al*. 2002). The uterus must recover rapidly after parturition in preparation for establishment of the next pregnancy. Recovery involves tissue repair, regeneration of the epithelium lining the endometrium, and elimination of bacteria that always contaminate the uterus around the time of parturition.

Microbial infections of the female reproductive tract

Escherichia coli infect the endometrium within a week of parturition, followed in subsequent weeks by *Trueperella pyogenes* and a range of anaerobic bacteria that include *Fusobacterium*, *Prevotella* and *Bacteriodes* species (Bonnett *et al*. 1991, Sheldon *et al*. 2002, 2010). Although not the focus of the present manuscript, it is important to note that bovine herpesvirus 4 is also tropic for endometrial stromal cells and causes *postpartum* uterine disease in some animals (Donofrio *et al*. 2007). The pathogens most consistently associated with uterine disease have been established by traditional microbiological culture methods. However, using molecular techniques, many more non-culturable microbes are identified in the uterus of *postpartum* cattle, with or without clinical signs of uterine disease (Santos & Bicalho 2012). Indeed, molecular markers for some of these species of bacteria may lead to diagnostic tests for a healthy or diseased uterus. The presence of several acid-producing strains of bacteria in the uterine lumen is interesting because *Lactobacilli* predominate in the healthy human vaginal microbiome, where they help to maintain a low pH to protect against pathogenic bacteria (Ravel *et al*. 2011).

Pathogens have evolved many virulence factors that may be important in the female reproductive tract, including toxins, proteases, neuramidases, secretion systems, fimbriae and iron acquisition systems. For example, endometrial pathogenic *E. coli* (EnPEC) adhere to and invade primary endometrial epithelial and stromal cells, cause inflammation, and recreate disease when infused into the uterus of mice (Sheldon *et al.* 2010). In particular, LPS appears to be the major virulence factor of EnPEC, stimulating TLR4-dependent inflammatory responses by endometrial cells (Sheldon *et al.* 2010, Cronin *et al.* 2012). On the other hand, the presence of *T. pyogenes* is associated with pus in the uterus, the severity of clinical signs and the extent of the subsequent subfertility (Bonnett *et al.* 1991, Westermann *et al.* 2010). The major virulence factor produced by all isolates of *T. pyogenes* is pyolysin, which is a heat-labile exotoxin that mulitmerises in cholesterol-rich domains of the plasma membrane of mammalian cells, creating transmembrane pores, 30–50 nm diameter, to disrupt ion balances and cause cytolysis (Jost & Billington 2005, Amos *et al.* 2014). Endometrial stromal cells are more sensitive to cytolysis by pyolysin than epithelial or immune cells, which explains how the bacteria act as pathogens, once the relatively resistant epithelium is denuded after parturition (Amos *et al.* 2014). Interestingly, the higher cholesterol content of stromal cells, compared with epithelial cells, explains the sensitivity of stromal cells to pyolysin, and reducing cellular cholesterol using cycoldextrin protects stromal cells against pyolysin (Amos *et al.* 2014).

Animal models of uterine infection and disease

The fundamental mechanisms of immunity and impact on the brain are most often studied by infusion of LPS into the uterus or into the peripheral circulation (Peter *et al.* 1989, Karsch *et al.* 2002). However, animal models of clinical uterine disease are also important because they are needed for the development of new vaccines or treatments, as well as uncovering the mechanisms of immunity in the female reproductive tract. Animal models of uterine disease have been generated in dairy and beef cattle, sheep and mice (Ayliffe & Noakes 1982, Karsch *et al.* 2002, Sheldon *et al.* 2010, Amos *et al.* 2014). Most of the animal models that replicate uterine disease rely on infusion of *E. coli* or *T. pyogenes* into the uterine lumen (Rowson *et al.* 1953, Ayliffe & Noakes 1982, Sheldon *et al.* 2010, Amos *et al.* 2014). A key step in generating models of uterine disease in cattle is mechanical debridement of the epithelium lining the endometrium (Ayliffe & Noakes 1982, Amos *et al.* 2014). Presumably, this allows *T. pyogenes* and pyolysin to access the stromal compartment of the endometrium to establish infection and cause tissue damage. The duration and severity of clinical endometritis following infusion of bacteria can be increased by administering exogenous progesterone to the animal, or by infusion of bacteria during the luteal phase of the oestrous cycle (Rowson *et al.* 1953, Lewis 2003). Similarly, early ovulation after parturition and establishment of a progesterone-dominated phase before pathogenic bacteria are eliminated predisposes to the development of pyometra (Olson *et al.* 1984). Conversely, administering oestrogens or oestrus often precludes the establishment of uterine disease (Rowson *et al.* 1953, Lewis 2003). However, the mechanisms linking the steroids to immunity are yet to be fully elucidated and are complex because there are interactions between the physiological effects of ovarian steroids and the inflammation caused by infusion of bacteria or LPS (Peter *et al.* 1989, Karsch *et al.* 2002, Lewis 2003).

Endometrial responses to bacteria in the uterus

Host responses to bacterial infection of the uterus are characterised by inflammation of the endometrium with infiltration by neutrophils and macrophages, and the accumulation of pus in the uterine lumen (Fig. 2). The diseases are defined as metritis, clinical endometritis or subclinical endometritis, depending on the signs and the time after parturition (Sheldon *et al.* 2006). The samples collected from the endometrium of diseased animals, compared with unaffected animals, have more abundant gene transcripts for the following: cytokines *IL1A, IL1B, IL6* and *TNF*; chemokines *CXCL8, CCL5* and *CXCL5*;

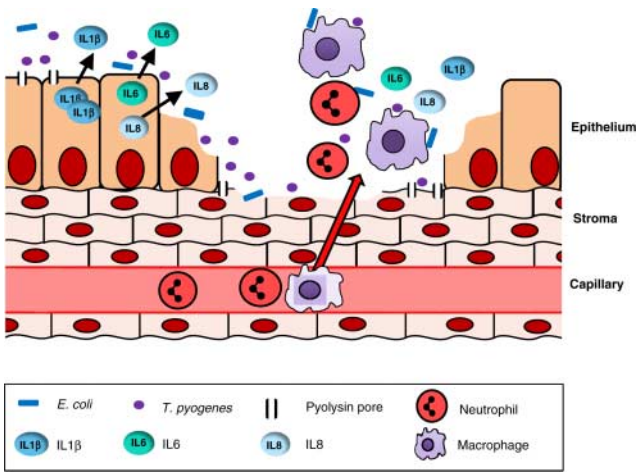

Figure 2 Pathogenic bacteria cause inflammation in the endometrium. After parturition, *Escherichia coli*, *Trueperella pyogenes* and other bacteria invade the uterus, and the pathogenic bacteria adhere to and invade the endometrium, particularly where there is tissue damage. *T. pyogenes* also produces pyolysin, which creates transmembrane pores in endometrial cells, causing cell death and further tissue damage. Endometrial epithelial and stromal cells sense pathogen-associated and damage-associated molecular patterns via innate immune receptors, such as TLRs, which leads to the release of cytokines and chemokines, such as IL1B, IL6 and IL8 (black arrows). These inflammatory mediators attract and activate haematopoietic cells, particularly neutrophils and macrophages, to the site of infection (red arrow), in order to clear the invading microbes and resolve the tissue damage.

calcium-binding proteins *S100A8, S100A9* and *S100A12* and receptors *TLR4* and *IL1R* (Gabler *et al*. 2009, Herath *et al*. 2009*b*, Wathes *et al*. 2009, Fischer *et al*. 2010). Furthermore, there are temporal changes in the expression of transcripts for *IL1B, IL6, CXCL8, TNF, CXCL5*, *HP* and *PTGS2* during the *postpartum* period, with peak expression on day 17 after parturition (Gabler *et al*. 2010). These temporal changes in gene expression reflect the histological evidence of inflammation during the first 3 weeks *postpartum*, which resolves by 40–60 days after parturition (Bonnett *et al*. 1991, Chapwanya *et al*. 2012). As well as increased gene expression, *postpartum* cows, particularly if the endometrium is infected, have increased protein abundance of S100A8 and S100A9 in the epithelium and stroma, as well as in immune cells, as determined by immunohistochemistry (Swangchan-Uthai *et al*. 2013). The findings at the transcription level are also supported by analysis of the proteome profile of cows with endometritis, which have more S100A9 and cathelicidin antimicrobial protein in the endometrium, compared with healthy cows (Ledgard *et al*. 2013). Taken together, these endometrial inflammatory responses are likely part of the physiological mechanisms of tissue homeostasis (Medzhitov 2008). However, stressors such as limitations on nutrient supply around the time of parturition may lead to excessive inflammatory responses and increased risk of disease (Wathes *et al*. 2009, Swangchan-Uthai *et al*. 2013).

The tissue responses following *postpartum* bacterial infection point to an important role for innate immunity. Endometrial biopsies collected from healthy and diseased *postpartum* dairy cows express mRNA for all ten TLRs (Herath *et al*. 2009*b*). In addition, *ex vivo* organ cultures of bovine endometrium accumulate IL1β, IL6, IL8, and prostaglandin E_2 when challenged with *E. coli* or *T. pyogenes* (Borges *et al*. 2012, Amos *et al*. 2014). Furthermore, pure populations of endometrial epithelial and stromal cells express most TLRs and their co-receptors, such as *MD2* and *CD14* (Herath *et al*. 2006, Davies *et al*. 2008). Addition of LPS to epithelial or stromal cells *in vitro* stimulates phosphorylation of MAPK and the nuclear translocation of NFκB, secretion of prostaglandin $F_{2\alpha}$, prostaglandin E_2, IL6 and IL8, and increased expression of antimicrobial peptides *MUC1*, *LAP* and *TAP* (Herath *et al*. 2006, Davies *et al*. 2008, Cronin *et al*. 2012). Intriguingly, the release of IL8 from polarised epithelial cells is vectorial and directed apically if the challenge is in the uterine lumen (MacKintosh *et al*. 2013). Similar cytokine and chemokine responses are also evident for cells treated with bacterial lipopeptides, with formal proof for the importance of innate immunity established using siRNA targeting *TLR1*, *TLR2*, *TLR4* and *TLR6* to suppress inflammation (Cronin *et al*. 2012, Turner *et al*. 2014). There are also increased peripheral blood concentrations of acute-phase proteins, such as serum amyloid A and haptoglobin, which are produced by hepatocytes in response to cytokines, such as IL6, from the infected uterus (Sheldon *et al*. 2001). Taken together, these data support the concept that the epithelial and stromal cells in the endometrium have roles in the initial sensing and response to pathogens (Fig. 2). A remaining question is the role of DAMPs in endometritis, because uterine infections are often associated with tissue damage including dystocia, retained foetal membranes and a large male calf.

Ovarian follicle responses to bacteria in the uterus

Follicle and oocyte development is a highly coordinated series of events starting with primordial-to-primary and secondary follicle transition, controlled by autocrine and paracrine signals including PTEN, C-KIT and FOXO3 (Parrott & Skinner 1999, Castrillon *et al*. 2003, Reddy *et al*. 2008). This is followed by gonadotrophin-dependent follicle growth, before formation of the follicle antrum and then selection of the dominant follicle. Whilst links between inflammation in the endometrium and infertility are intuitive, it was less obvious whether uterine infections might affect ovarian function. However, in dairy cattle, uterine disease is associated with LPS in follicular fluid, slower growth of dominant follicles, lower peripheral plasma concentrations of oestradiol and increased risks of anoestrus or cystic ovarian disease (Opsomer *et al*. 2000, Sheldon *et al*. 2002, Herath *et al*. 2007). The mechanisms underlying these observations are now emerging.

During early stage of follicle development in the bovine ovary, LPS increases *ex vivo* primordial-to-primary follicle activation, which is associated with loss of primordial follicle PTEN and cytoplasmic translocation of FOXO3 (Bromfield & Sheldon 2013). The implication of this work is that multiple bacterial infections could deplete the ovarian follicle reserve, although LPS does not affect secondary follicle growth and function (Bromfield & Sheldon 2013). Antral follicles present a special situation because whilst the stroma of the ovary contains a wide range of immune cells including macrophages that might protect pre-antral follicles, healthy antral follicles are devoid of such cells within the basement membrane (Bromfield & Sheldon 2011). Intriguingly, mural granulosa cells lining emerged and dominant antral follicles express TLRs and secrete IL1β, IL6 and IL8 when treated with ligands that bind TLR2 or TLR4 (Bromfield & Sheldon 2011, Price & Sheldon 2013, Price *et al*. 2013). Treatment with PAMPs also reduces the expression of *CYP19A1* mRNA and aromatase protein, as well as the secretion of oestradiol from granulosa cells (Herath *et al*. 2007, Price *et al*. 2013). The responses of granulosa cells are rapid, with increased phosphorylation of p38 and ERK1/2, and expression of *IL6, IL1B, IL10, TNF, CXCL8* and *CCL5* mRNA (Bromfield & Sheldon 2011, Price & Sheldon 2013, Price *et al*. 2013). Furthermore, the innate immune response is attenuated using siRNA targeting *TLR2* or

TLR4, or by treating granulosa cells with inhibitors targeting MAPK or NFκB.

As physiological events within the ovary use several inflammatory mediators, events associated with infection of the female genital tract may disrupt the biology of reproduction. For example, expansion of the cumulus–oocyte complex is a key event during ovulation but, even in the absence of gonadotrophin, high concentrations of LPS cause inappropriate expansion, which would compromise fertility (Bromfield & Sheldon 2011). Even the oocyte is at risk from pathogens, and bovine virus diarrhoea and bovine herpesvirus 1 are amongst viruses that have been isolated from oocytes denuded of granulosa cells. Furthermore, *in vitro* maturation of oocytes in the presence of LPS reduces meiotic competence from 85 to 64%, and this failure cannot be rescued by the administration of exogenous gonadotrophins (Bromfield & Sheldon 2013). Therefore, alterations in the follicular environment due to pathogen exposure results in diminished oocyte quality, further affecting fertility.

Host responses in the brain associated with uterine disease

In *postpartum* cows, LPS in the uterus suppresses the LH surge and prevents ovulation (Peter *et al*. 1989). Also, during the follicular phase, LPS decreases LH pulse frequency, decreases oestradiol and interrupts the LH surge and ovulation in cattle (Suzuki *et al*. 2001, Lavon *et al*. 2008). Similar observations have been made in sheep, which is the species most often used to study the effect of LPS in the ruminant brain (reviewed by Karsch *et al*. (2002)). Neuroendocrine activity at the hypothalamic level is inhibited by LPS suppressing both the frequency and amplitude of GNRH/LH pulses. However, there are also effects within the pituitary gland as LPS lowers LH responses to exogenous GNRH (Karsch *et al*. 2002). Indeed, treatment of ewes with LPS suppresses the expression of genes encoding GNRH receptor (GNRHR) and LHβ (Herman *et al*. 2013*b*). However, the peripheral plasma concentrations of follicle-stimulating hormone are unaffected by uterine disease in cattle (Sheldon *et al*. 2002).

The mechanisms linking LPS administration to changes in the hypothalamus and pituitary are elusive. One possibility is that increased circulating cortisol, associated with the stress of LPS treatment, modulates brain function. However, whilst exogenous cortisol suppresses pulsatile LH secretion in ewes, LPS still inhibits GNRH and LH pulsatility when endogenous cortisol secretion is inhibited (Karsch *et al*. 2002). Opioids are involved in LPS suppression of LH because an opioid receptor antagonist increases LH responses to LPS in heifers (Kujjo *et al*. 1995). However, the same opioid receptor antagonist does not alter the reduction in LH in response to acute bacteraemia (Leshin & Malven 1984). A similar conundrum is evident using inhibitors of prostaglandin synthesis, which counter the LPS-induced suppression of LH pulses but not the LPS-induced blockage of the LH surge (Karsch *et al*. 2002, Breen *et al*. 2004). Another approach to understanding how LPS affects hypothalamic activity is to consider innate immunity. Preliminary results show that LPS increases the expression of *TLR4* genes in the medial preoptic area (mPOA), anterior hypothalamus, medial basal hypothalamus (comprising the arcuate nucleus (ARC) and ventromedial nucleus (VMN)) and median eminence (Fig. 3; Herman *et al*. 2013*a*). Peripheral LPS also increases mRNA levels of *FOS* and *IL1B* in the ventricular system, which is the choroid plexus in the brain endothelium (Vellucci *et al*. 1995); and administration of IL1β into the ventricular system suppresses expression of *GNRHR* and *LHB* genes in the sheep pituitary (Herman *et al*. 2012). Therefore, LPS and inflammatory mediators may act centrally to inhibit neuroendocrine events; although there needs to be some caution because IL1β and TNFα also act as normal neuromodulators within the hypothalamus.

Administration of LPS during the follicular phase causes a delay in the onset of LH surge and this is positively correlated with the duration of oestradiol signal disruption (Fergani *et al*. 2012). Also, the expression of *GNRH* and *GNRHR* mRNA levels in the mPOA and median eminence is lower, but unexpectedly not in the medial basal hypothalamus (Herman *et al*. 2012). These neuroendocrine disruptions are associated with an increase in *C-FOS* within the VMN, ARC, mPOA and diagonal band of Broca, but there is no anticipated activation of oestradiol receptor (ERα) containing cells in the ARC and mPOA (Fig. 3; Fergani *et al*. 2012). LPS also inhibits kisspeptin and dynorphin cell activation in the ARC and kisspeptin in the mPOA, and increases somatostatin activation in the VMN (Fergani *et al*. 2013); and C Fergani, J E Routly, D N Jones, L C Pickavance, R F Smith and H Dobson (2011), unpublished data). Somatostatin is one of the most potent inhibitors of GNRH neurone excitability so far identified (Bhattarai *et al*. 2010).

Not surprisingly, LPS treatment increases *FOS* and corticotrophin-releasing factor (*CRF*) mRNA levels within the paraventricular nucleus, the stress-control centre of the hypothalamus (Vellucci *et al*. 1995), as well as increasing CRF receptors (CRFR) in the ARC and median eminence (Fig. 3; Fergani *et al*. 2013). Therefore, there are two possible pathways for CRF suppression of GNRH, one being the direct association of CRF and GNRH cell terminals in the ME (Ghuman *et al*. 2010) and the other being the regulation of kisspeptin-neurokinin B-dynorphin cells and others in the ARC and median eminence (Fergani *et al*. 2013).

A cholinergic pathway is also involved in restraining the central response to LPS. Peripheral LPS increases the expression of *IL1B* and *IL1R1* genes, as well as IL1β concentration in the mPOA and the hypothalamus, but the effect is reversed by concomitant rivastigmine, an

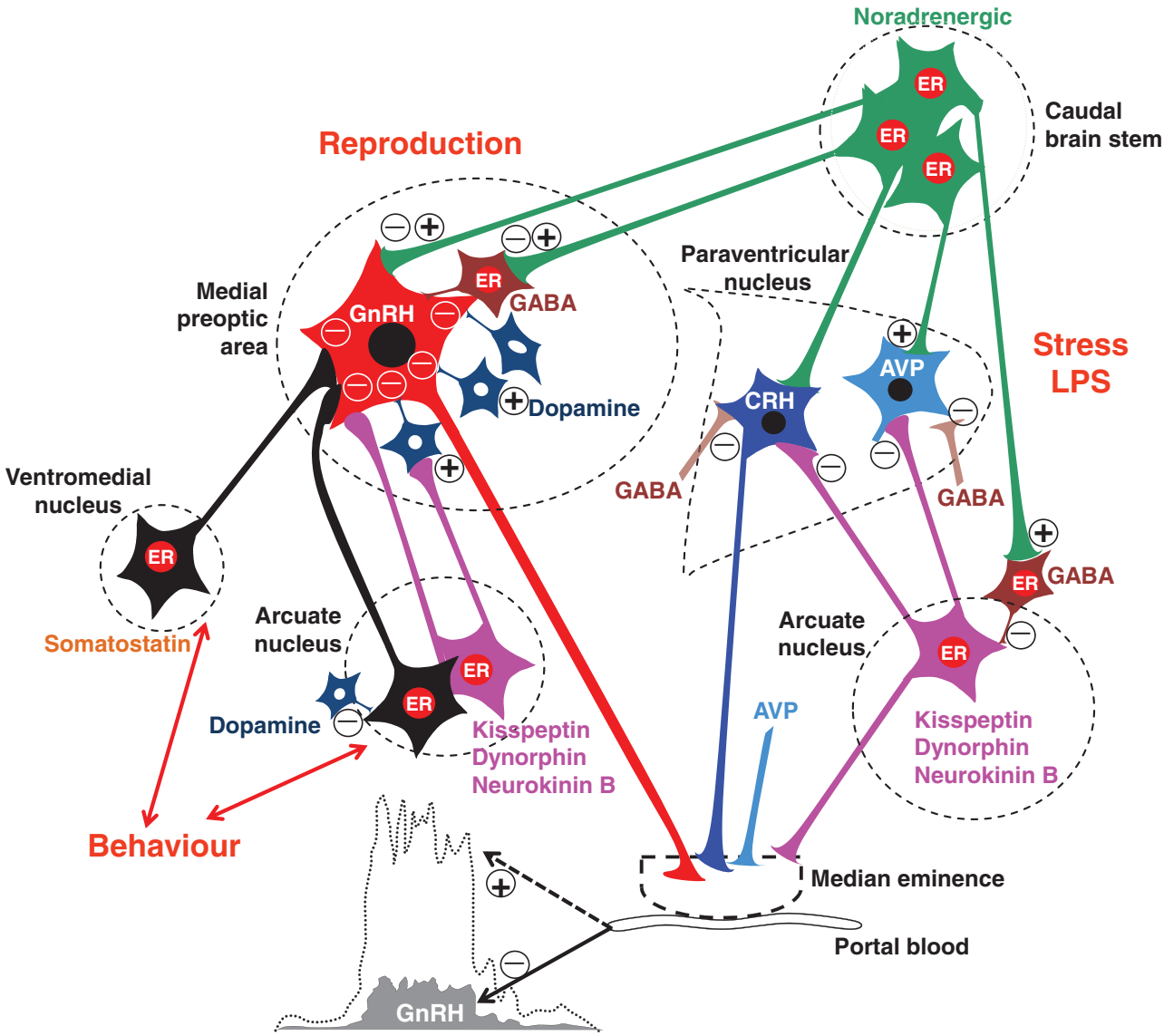

Figure 3 Interaction between neurones involved in reproduction, behaviour and stress. Noradrenergic cells in the ovine brain stem project to both the preoptic area (reproduction) and the paraventricular nucleus (stress centre); LPS activates neurones in the latter. Changes in activity of β-endorphin and dynorphin neurones of the arcuate nucleus influence the paraventricular nucleus and medial preoptic area output. In the arcuate nucleus, the activities of oestradiol receptor (ER)α-positive neurones (kisspeptin/dynorphin/neurokinin B cells) are modulated by LPS, as are the somatostatin cells in the ventromedial nucleus. In the median eminence, corticotrophin-releasing hormone (CRH), but not arginine vasopressin (AVP) terminals, and kisspeptin/dynorphin/neurokinin B terminals are in close contact with GNRH terminals, possibly providing another site for the disruption of GNRH release; the normal profile of GNRH release (dotted line) is suppressed during stress (grey infill). Neural activation and suppressive influences are indicated by + and − symbols respectively. Adapted from original drawing by SPS Ghuman (Dobson H, Fergani C, Routly JE & Smith RF 2012 Effects of stress on reproduction in ewes. *Animal Reproduction Science* **130** 135–140. doi:10.1016/j.anireprosci.2012.01.006), and reproduced with permission.

acetylcholinesterase inhibitor (Herman *et al.* 2012). Indeed, acetylcholine itself reduces LPS-induced release of IL1β and TNFα. Furthermore, rivastigmine reverses LPS-induced suppression of GNRH and GNRH-R mRNA levels in the ME as well as *LHB* and *GNRHR* mRNA levels in the pituitary (Herman *et al.* 2013*b*).

A final caveat is that many of the GNRH/LH responses are not specific to LPS. Similar changes occur following other stressors, for example shearing, transport and isolation (Dobson *et al.* 2012). Thus, future hypotheses must take into account similarities and differences in responses, as well as issues relating to stressors of differing intensity.

Conclusions

The integration of innate immunity and inflammation in the female reproductive system has likely evolved

over millennia. There appears to be adoption of inflammatory mediators in the physiology of reproduction in cattle, whilst innate immunity remains important for maintenance of tissue homoeostasis and defence against pathogens. In dairy cattle, the need to control *postpartum* infections of the genital tract highlights the importance of understanding the mechanisms underlying the integration of innate immunity, inflammation and reproductive physiology. In addition, these interactions are modulated by stress, the environment and metabolism, which are likely to become more important with the need to feed the expanding human population and to counter climate change. An integrated approach to understand the interactions between innate immunity, inflammation and reproduction will be important for developing strategies to maintain animal health and welfare.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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