REVIEW

Mechanisms linking bacterial infections of the bovine endometrium to disease and infertility

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Abstract

Bacterial infections of the endometrium after parturition commonly cause metritis and endometritis in dairy cattle, and these diseases are important because they compromise animal welfare and incur economic costs, as well as delaying or preventing conception. Here we highlight that uterine infections cause infertility, discuss which bacteria cause uterine disease, and review the evidence for mechanisms of inflammation and tissue damage in the endometrium. Bacteria cultured from the uterus of diseased animals include *Escherichia coli*, *Trueperella pyogenes*, and several anaerobic species, but their causative role in disease is challenged by the discovery of many other bacteria in the uterine disease microbiome. Irrespective of the species of bacteria, endometrial cell inflammatory responses to infection initially depend on innate immunity, with Toll-like receptors binding pathogen-associated molecular patterns, such as lipopolysaccharide and bacterial lipopeptides. In addition to tissue damage associated with parturition and inflammation, endometrial cell death is caused by a cholesterol-dependent cytolysin secreted by *T. pyogenes*, called pyolysin, which forms pores in plasma membranes of endometrial cells. However, endometrial cells surprisingly do not sense damage-associated molecular patterns, but a combination of infections followed by cell damage leads to release of the intracellular cytokine interleukin (IL)-1 alpha from endometrial cells, which then acts to scale inflammatory responses. To develop strategies to limit the impact of uterine disease on fertility, future work should focus on determining which bacteria and virulence factors cause endometritis, and understanding how the host response to infection is regulated in the endometrium.

Key Words

Cow, Endometritis, Cytolysis, Innate immunity, Uterus.

1. Introduction

Bacterial infections of the uterus cause disease and infertility in dairy cattle, particularly after parturition, when they lead to metritis and endometritis in up to 40% of animals [1]. These postpartum uterine diseases are important because they compromise animal welfare, and incur costs for treatment, reduced milk production, and replacement of infertile animals; €1.4 billion every year in the EU alone [2]. Here we address the question of how infections of the endometrium are linked to disease and infertility. We highlight the impact of uterine disease on fertility, then discuss which bacteria cause disease, and finally assimilate recent evidence about the mechanisms of inflammation and tissue damage in the endometrium.

2. Bacterial infections of the uterus cause infertility

In a meta-analysis of more than 10,000 animals, postpartum metritis increased the time to first insemination by 7.2 days, reduced conception rate to first insemination by 20%, and increased the calving to conception interval by 18.6 days [3]. Similarly, clinical endometritis increased the interval to first insemination by 11 days, and delayed conception by 32 days, compared with animals that did not have endometritis [4]. Cows with clinical endometritis between 20 and 33 days post partum were also 1.7 times more likely to be culled for reproductive failure than cows without endometritis [5]. Furthermore, infertility could be due to the endometrial inflammation in postpartum cattle with *T. pyogenes* [6]. Together, these observations linking bacterial infections of the uterus with infertility provide an impetus to discover the underlying mechanisms. Evidence for how uterine disease impacts ovarian and neuroendocrine function have been reviewed recently [7]. So, the objective of the present review is to focus on the uterus.

3. Microbial infections of the postpartum uterus

From a historical perspective, microbial disease of the uterus of cattle merited little comment 80 years ago, and endometritis was not considered a common problem. However, between 1960 and 2000, endometritis in cattle started to be the subject of investigations to

understand the pathogenesis of the disease and to select the most effective treatments. In one study, 93% of the uteri obtained within 15 days of calving yielded bacteria on aerobic and anaerobic culture of endometrial swabs and tissue [8]. The proportion of uteri from which bacteria were isolated had declined to 78% by 16 to 30 days, 50% by 31 to 45 days, and 9% by 46 to 60 days postpartum. Similar proportions of animals yielded culturable bacteria in subsequent studies [9, 10]. However, the situation is more complicated because the bacterial flora fluctuates during the first 7 weeks postpartum due to spontaneous contamination, clearance and recontamination [11]. Furthermore, which bacterial isolates are contaminants of the uterus and which are pathogens is open to debate. Uterine infection was most commonly associated with the presence of *Escherichia coli, Trueperella pyogenes, Fusobacterium necrophorum*, and *Prevotella* or *Bacteroides* species in studies spanning from the 1960's to the 1990's [8, 10-12]. In the last 15 years, studies using aerobic and anaerobic culture methods provided similar evidence to the earlier investigations [9, 13-15]. These bacteria were identified by standard culture techniques and are classified into pathogens, potential pathogens and opportunist contaminants (Table 1). In particular, *T. pyogenes* is linked to the severity of endometrial pathology and clinical disease [10, 14, 16]. Furthermore, *T. pyogenes, F. necrophorum* and *Prevotella* species can act synergistically to increase the likelihood of endometritis and the severity of disease [17, 18]. Associations between uterine disease and bacteria that are not readily cultured by standard techniques emerged recently as researchers started to use biochemical, molecular and sequencing techniques [19-23]. These studies have provided some contention in the field. Whilst some of the studies found *E. coli*, *T. pyogenes* and the expected anaerobic bacteria; others, surprisingly, found no evidence from their molecular analyses that *E. coli* or *T. pyogenes* cause uterine disease but rather reported finding other *Fusobacteria* species*, Bacteroidetes* and *Firmicutes* (Table 1). Gaps in knowledge remain about the relative contribution of culturable compared with "uncultureable" bacteria to uterine disease, and synergistic interactions between *E. coli* or *T. pyogenes* and other bacteria. A consistent finding among most molecular microbiology studies is that anaerobic bacteria are abundant in diseased

uteri [19, 20]. Perhaps this is not surprising as the endometrium is a microaerophilic environment, and tissue damage and necrosis likely reduce the oxygen tension further, as well as the endometrium providing nutrients to facilitate bacterial growth. Indeed, the risk factors most frequently associated with uterine infection are stillbirth, twins, dystocia, caesarean section operation, and particularly retained foetal membranes [24-27].

Direct links between microbes and uterine disease remain to be explored, especially for unculture able microbes. However, an experimental vaccine containing components of *E. coli*, *F. necrophorum* and *T. pyogenes*, prevented metritis in dairy cows [28]. Furthermore, infusion of *E. coli* and *T. pyogenes* into the uterus of naive cows induces endometritis [29, 30]. So, taking the combined evidence for *E. coli* and *T. pyogenes* playing roles in postpartum uterine disease in dairy cattle, we will focus on these two microbes (Fig. 1).

3.1 *Escherichia coli*

It was assumed that *E. coli* isolated from the uterine lumen was associated with fecal contamination of the uterus during parturition, even though the level of fecal contamination on farms did not affect the uterine microbiome or severity of endometritis [12, 27]. However, molecular typing techniques found that uterine disease is associated with phylogenetic clusters of *E. coli* [31, 32]. These endometrial pathogenic *E. coli* (EnPEC) tend to be phylogenetically distant from the majority of extra-intestinal pathogenic *E. coli* (ExPEC), and more closely related to human intestinal pathogens [33]. However, the EnPEC have acquired, via horizontal transfer, DNA encoding iron acquisition systems and a virulence plasmid, similar to that found in several ExPECs. On the other hand, EnPEC have few virulence factors typical of enteric *E. coli*, although they possess the gene encoding the *FimH* adhesion factor, and FimH fosters adhesion of EnPEc to endometrial cells [31, 32]. Beyond adhesion, EnPEC exploit cellular microfilaments and microtubules to invade endometrial cells [31]. Of course, like all Gram-negative bacteria, lipopolysaccharide (LPS,

endotoxin) is a principal component of the bacterial cell wall of EnPEC, and is an important virulence factor [31].

3.2 *Trueperella pyogenes*

Trueperella pyogenes has undergone several name changes in the last 60 years, from *Corynebacterium pyogenes*, via *Actinomyces pyogenes* and *Arcanobacterium pyogenes*, to *T. pyogenes* [34]. *Trueperella pyogenes* is consistently associated with the severity of postpartum endometritis in cattle [14, 16, 22], and causes uterine disease when infused into the uterus of cattle [30, 35]. Unlike the diverse strains of *E. coli*, uterine isolates of *T. pyogenes* are phylogenetically similar [30]. Although, there is some variation in expression of virulence factors, including: neuraminidases *nanH* and *nanP*, which cleave sialic acids from host molecules, exposes host cell receptors for increased adhesion, and reduce mucus viscosity [36-38]. The collagen-binding protein *cbpA*, which binds to collagen types I, II and IV, and promotes adhesion to collagen-rich tissues; and *fimA*, required for fimbrial biogenesis and attachment of *T. pyogenes* to mucosal surfaces [36-38].

However, the most striking finding is that the *plo* gene is expressed ubiquitously by all isolates of *T. pyogenes* [30, 36, 37]. The *plo* gene encodes a cytolysin, pyolysin, which is the virulence factor thought to be responsible for much of the pathology associated with *T. pyogenes* infections, including mastitis in cattle, and abscesses in pigs, goats, cattle and mice [38, 39]. The *plo* gene sequence is identical and the *plo* gene promoter is highly similar amongst uterine isolates of *T. pyogenes* [30]. Pyolysin is a cholesterol-dependent cytolysin, which is secreted as a monomeric protein by *T. pyogenes* [38, 39]. Lipid rafts are membrane microdomains that are enriched in cholesterol, sphingomyelin, sphingolipids and phospholipids, and pyolysin inserts into these cholesterol-rich domains in the plasma membrane of host cells, and pyolysin aggregates to form 30 nm diameter pores, leading to osmotic cell death (Fig. 1). Surprisingly, bovine endometrial stromal cells are more sensitive to pyolysin-mediated cytolysis than epithelial cells, neutrophils, monocytes or lymphocytes

[30]. The sensitivity of stromal cells to pyolysin provides an explanation for how *T. pyogenes* causes cytolysis in the endometrium, once the protective epithelium is lost after parturition. Furthermore, loss of the epithelium likely allows the bacterium to transit from a commensal state to act as a pathogen. The sensitivity of endometrial stromal cells to pyolysin may be because stromal cells contain more cholesterol than epithelial cells [30]. Indeed, if stromal cell cholesterol content is reduced using methyl-β cyclodextrin, the stromal cells become as resistant to pyolysin as endometrial epithelial cells [30, 40]. Similarly, the dynamin inhibitor Dynasore, which inhibits endocytosis from the plasma membrane, also reduces endometrial cell cholesterol and disperses plasma membrane lipid rafts, which protects against pyolysin [40]. In addition to changes in plasma membrane cholesterol, the physical properties of cell membranes and the shape of cells may be modulated. For example, Dynasore destabilizes and remodels F-actin, which may influence the distribution of cholesterol in plasma membranes and membrane biophysical properties [41]. However, further work is needed to determine the specific protective effects in endometrial cells.

4. Immunity, inflammation and tissue damage

As well as tissue damage associated with parturient problems, and the cell death caused by bacterial pore-forming toxins, much of the pathology in the postpartum endometrium is a consequence of the host innate immune response to bacteria.

4.1 Innate immunity in the endometrium

The innate immune response is predicated on cellular pattern recognition receptors that bind pathogen-associated molecular patterns (PAMPs), which are components of microbes but not eukaryotic cells [42]. Evidence for pattern recognition receptors was first uncovered using *Drosophila melanogaster*, where *Toll* was found to be required for protection against fungal infection [43]. The first functional mammalian Toll-like receptor (TLR) was identified in mutant mice, where TLR4 protected against infection with Gram-negative bacteria by recognizing LPS [44]. A range of pattern recognition receptors have now been discovered,

168 with some expressed on the surface of hematopoietic immune cells, whilst others are
169 located in the cytoplasm [42]. Binding of PAMPs to pattern recognition receptors induces
170 intracellular signaling, often including mitogen-activated protein kinases and nuclear factor
171 kappa B, leading to the secretion of cytokines such as interleukin (IL)-1β and IL-6,
172 chemokines such as IL-8, prostaglandins, and antimicrobial peptides [42].
173
174 Pattern recognition receptors are not only present in hematopoietic immune cells, but also in
175 endometrial epithelial and stromal cells. Bovine endometrial cells use TLR4 to sense LPS,
176 and TLR1, TLR2 and TLR6 to detect bacterial lipopeptides, leading to the secretion of
177 interleukin (IL)-8, IL-6 and prostaglandin E_2 (Fig. 1) [45-48]. These *in vitro* responses mimic
178 the effect of EnPEC and *T. pyogenes* on *ex vivo* organ cultures of endometrium, which
179 stimulates the accumulation of IL-8 and IL-6, as well as IL-1β [49, 50]. In addition,
180 prostaglandin E_2 is more abundant in animals with uterine disease, and changes in
181 prostaglandin secretion might impact the physiological control of ovarian function [48]. The *in*
182 *vitro* inflammatory responses by endometrial cells also reflect increased abundance of genes
183 encoding inflammatory mediators in endometrium collected from animals with uterine
184 disease. The upregulated genes in diseased endometrium, or in cytology samples collected
185 from the endometrium, include cytokines such as *IL1A, IL1B* and *IL6*; chemokines such as
186 *CXCL5* and *CXCL8*; and antimicrobial peptides such as *TAP, DEFB5* and *DEFB1* [51-55].
187 However, metabolic deficits after parturition, often termed “negative energy balance”, may
188 perturb immunity and the ability to clear microbes from the endometrium [56].
189
190 Chemokines, such as IL-8 (encoded by the *CXCL8* gene), attract neutrophils and
191 macrophages to the site of infection or tissue damage, where these hematopoietic cells are
192 regulated by cytokines, such as IL-6 [57]. In the postpartum uterus the epithelium is often
193 disrupted, with migration of neutrophils and macrophages through the stroma (Fig. 1).
194 However, it is interesting to note that IL-6 is only secreted apically by the polarized epithelial
195 cells of a confluent epithelium *in vitro* [58]. Presumably, IL-6 is secreted apically into the

uterine lumen to ensure immune cells are only exposed to IL-6 once they reach the lumen, unless the epithelium is breached. Furthermore, a confluent epithelium also protects the underlying stroma from PAMPs, and from bacterial toxins such as pyolysin [58].

4.2 Tissue damage and inflammation in the endometrium

The “danger hypothesis” extends the role of innate immunity to suggest that cells also use pattern recognition receptors to sense and respond to signals from damaged tissues [59, 60]. Damage-associated molecular patterns (DAMPs) are released into the extracellular fluid by damaged or necrotic cells, or from the extracellular matrix [59, 60]. In a similar manner to the responses to PAMPs, DAMPs such as the nuclear protein high molecular group box 1 (HMGB1) are released by necrotic cells or activated macrophages, and bind to receptors including TLRs to stimulate inflammation [61, 62]. Hyaluronan is an example of an extracellular matrix DAMP produced during tissue damage, which stimulates inflammation via TLR2 and TLR4 [63]. As there is considerable tissue damage in the postpartum endometrium, it was surprising that bovine endometrial cells or mononuclear cells did not respond to HMGB1 or a range of different molecular weights of hyaluronan [64]. Instead, the endometrium exploits an alternative danger-sensing mechanism. Damaged cells passively release the intracellular cytokine IL-1α [65, 66]; and, endometrial cells release IL-1α protein if there is a combination of exposure to bacteria, or bacterial PAMPs, followed by cell damage (Fig. 1) [64]. In turn, IL-1α binds the cognate IL-1R on adjacent endometrial cells to stimulate the production of inflammatory mediators such as IL-6. Thus, IL-1α scales the inflammatory response in the endometrium when there is infection followed by cell damage. This mechanism makes biological sense, as the intensity of the inflammatory response in a tissue should match the severity of the challenge [67].

5. Prospects

The rise in incidence and economic impact of postpartum uterine disease in dairy cattle over the last 40 years was unexpected, and few of the discoveries about endometritis discussed

in the present review were predictable even 20 years ago. Although, a remaining open question is the role of unculturable bacteria, the main pathogens were identified by culture and recent evidence shows that they are well adapted to the endometrium. The role of innate immunity for sensing and responding to pathogen-associated molecular patterns in endometritis is now clearly established. We have also uncovered IL-1α as a key damage-associated molecular pattern, when there is damage and infection of the endometrium. Finally, there is emerging evidence for the role of virulence factors, such as the pore-forming toxin pyolysin. Future work should focus on determining which bacteria and virulence factors cause endometritis, and understanding how the host response to infection is regulated in the endometrium. New knowledge about postpartum uterine disease will provide a platform for new therapeutics and vaccines.

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Figure legend

Figure 1

Mechanisms of pathology in the postpartum endometrium

Uterine infection is commonly associated with the presence of Gram-negative *E. coli*, and Gram-positive *T. pyogenes* and anaerobes. (A) Pattern recognition receptors, such as the Toll-like Receptor (TLR) family, on endometrial cells detect pathogen-associated molecular patterns (PAMPs), leading to the secretion of cytokines, such as IL-1β, IL-6 and IL-10, chemokines, such as IL-8 and CCL5, and prostaglandin E_2. Chemokines attract neutrophils and macrophages to the site of infection, where they are regulated by cytokines such as IL-6. (B) The cholesterol-dependent cytolysin - pyolysin (PLO) is secreted as a monomeric protein from *T. pyogenes*, and inserts into cholesterol-rich lipid rafts in the plasma membrane of host cells, forming pores that lead to osmotic cell death. Loss of the protective epithelium, often through damage incurred during parturition or from PLO, allows bacterial access to the underlying stromal cells. The stromal cells are more sensitive to pyolysin-mediated cytolysis as their plasma membrane contains more cholesterol than epithelial cells. In addition, activation of TLRs on stromal cell induces further production of cytokines and chemokines. (C) Following infection, damaged epithelial or stromal cells release the damage-associated molecular patterns (DAMPs) IL-1α, which signal through the cognate receptor IL-1R1 on epithelial and stromal cells to scale the inflammatory response.

446 **Table 1**

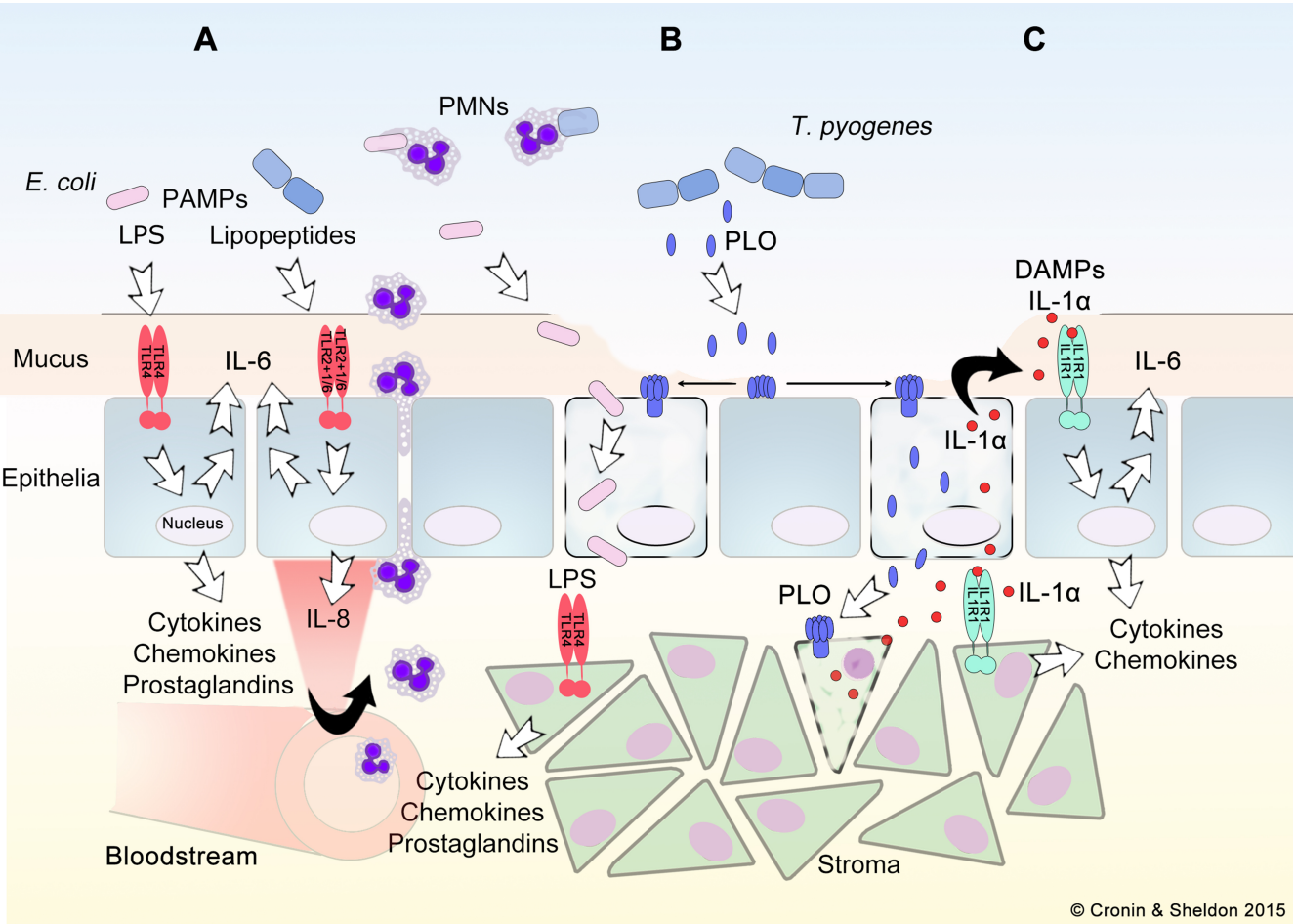
447 Categorization of bacteria, isolated by aerobic and anaerobic culture of uterine swabs,
448 based on their potential pathogenicity [8-14, 17, 18]. Categories: (1) pathogens known to
449 cause endometrial lesions; (2) potential uterine pathogens; and, (3) bacteria not recognized
450 as uterine pathogens that are likely contaminants of the uterine lumen

451

Pathogens	Potential pathogens	Contaminants
Escherichia coli	*Acinetobacter* spp.	*Aerococcus viridans*
Trueperella pyogenes	*Bacillus licheniformis*	*Clostridium butyricum*
Prevotella spp.	*Enterococcus faecalis*	*Clostridium perfringens*
Fusobacterium necrophorum	*Haemophilus somnus*	*Corynebacterium* spp.
Fusobacterium nucleatum	*Mannhiemia haemolytica*	*Enterobacter aerogenes*
	Pasteurella multocida	*Klebsiella pneumoniae*
	Peptostreptococcus spp.	*Micrococcus* spp.
	Staphylococcus aureus	*Providencia rettgeri*
	(coagulase +)	*Providencia stuartii*
	Streptococcus uberis	*Proteus* spp.
	Bacteroidetes species	*Proprionobacterium granulosa*
	Firmicutes species	*Staphylococcus* species
	Fusobacteria species	α-haemolyic Stretococci
		Streptococcus acidominimus

452

453


A
B
C
PMNs
T. pyogenes
E. coli
PAMPs
LPS
Lipopeptides
PLO
DAMPs
IL-1α
Mucus
TLR4
TLR4
TLR2+1/6
TLR2+1/6
IL-6
IL-1R1
IL-1R1
IL-6
IL-1α
Epithelia
Nucleus
Cytokines
Chemokines
Prostaglandins
IL-8
LPS
TLR4
TLR4
PLO
IL-1R1
IL-1R1
IL-1α
Cytokines
Chemokines
Cytokines
Chemokines
Prostaglandins
Bloodstream
Stroma
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