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Tolerance and innate immunity shape the development of postpartum uterine disease and the impact of endometritis in dairy cattle

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ABSTRACT

Bacteria are ubiquitous in the uterus of cattle after parturition, but 50 years ago, cows tolerated these bacteria and few animals developed uterine disease. Now, up to 40% of dairy cattle develop postpartum uterine disease. Uterine disease causes infertility by compromising not only the function of the endometrium but also the ovary. Animals defend themselves against pathogens using tolerance and resistance mechanisms. Tolerance is the ability to limit the disease severity induced by a given pathogen burden. Resistance is the ability to limit the pathogen burden, and is usually the function of immunity. Cellular barriers, mucus, and tissue repair contribute to tolerance in the endometrium. However, once tolerance is overcome, endometrial cells also have roles in resistance to pathogens, with innate immunity driving the production of cytokines, chemokines and prostaglandins. We propose that failures in endometrial tolerance to pathogenic bacteria and the subsequent innate immune response shape postpartum uterine disease.

INTRODUCTION

Bacteria are ubiquitous in the uterus of dairy cattle after parturition, and invasion and growth of bacteria in the endometrium causes postpartum uterine disease (1). Fifty years ago, dairy cows tolerated these bacteria in the uterus and animals only occasionally developed uterine

disease. Now, up to 40% of dairy cattle develop postpartum uterine disease every year, ranging from subclinical endometritis to life-threatening metritis (Figure 1A). These uterine diseases compromise animal health, welfare, and productivity. In particular, endometritis impairs the fertility of dairy cattle by generating a hostile environment for the embryo in the female genital tract, and by perturbing ovarian follicle function and oocyte health. Emergence of endemic postpartum uterine disease has coincided with intensification of farming, and breeding dairy cattle to increase milk yields by five-fold over the last 50 years. The association between uterine disease and dairy production is a concern because intensification of dairy farming will continue, with the milk yield of cows in the USA projected to double in the next 50 years (2).

Bacteria associated with uterine disease employ virulence factors that cause tissue damage and provoke inflammation in the endometrium. The ability of an organism to counter pathogenic microbes, or the animal's resilience, depends on **resistance** and **tolerance** {see **SIDEBAR**} (3-5). Tolerance is the ability to limit the disease severity induced by a given pathogen burden (3, 5, 6). Resistance is the ability to limit the pathogen burden, and is usually the function of immunity. Together tolerance and resistance determine the severity of disease. Here, we argue that tolerance to bacteria invading the endometrium is more important than resistance for the development of postpartum uterine disease. If we are correct, this would imply that antibiotics used to treat uterine disease by killing pathogens would have little benefit for health or fertility. Instead, the aim should be to enhance animal tolerance to uterine pathogens, and to control risk factors that compromise tolerance. In this review, we outline the fundamental aspects of the postpartum period and uterine disease, and then discuss how tolerance and resistance shape the development of postpartum uterine disease, and the impact of endometritis on dairy cattle.

THE POSTPARTUM PERIOD

The events that are required to foster optimal fertility after parturition and expulsion of the placenta are:

1. Prompt involution of the uterus and restoration of a receptive endometrium.

2. Return of ovarian cyclical activity and ovulation of competent oocytes.

3. Efficient control of bacteria in the uterus.

Several factors influence uterine involution, including expulsion of the placenta, postpartum uterine muscular contractions, turnover of the extracellular matrix, and regeneration of the uterine caruncles. During the first 30 days post partum, the weight of the uterus decreases from about 9 kg at parturition to about 1 kg, and the uterine horns return to their non-pregnant dimensions (7, 8). The epithelium of the endometrium is often damaged during parturition, and inflammation, remodeling, and regeneration of the endometrium is part of postpartum physiology (7, 9). The caruncular tissue undergoes necrosis of the stratum compactum within 7 days post partum, with the tissues sloughing by 14 days, followed by re-epithelialization of the caruncles by 30 days (9). As epithelialization progresses, the initially flattened epithelial cells develop their columnar appearance, typical of normal endometrium. Neutrophils and lymphoid aggregates are common in the tissue during the repair of the endometrium, and they are likely to be part of the response to tissue damage, as well as to the presence of microbes.

The return of ovarian cyclic activity after parturition requires a coordinated endocrine program by the hypothalamus, pituitary, ovary, and uterus (10-12). Briefly, within days of parturition, the circulating steroid hormone concentrations associated with pregnancy decrease to basal values. About 7 days post partum there is an increase in plasma follicle stimulating hormone (FSH) concentration, emergence of a cohort of growing follicles, and selection of the first postpartum dominant follicle about 10 days after parturition, with subsequent recurrent increases in FSH and emergence of waves of growing follicles every 7 to 10 days. The first postpartum dominant follicle may ovulate to form a corpus luteum, marking the return of ovarian cyclic activity; or the dominant follicle may undergo atresia with emergence of subsequent dominant follicles typical of postpartum anestrus; or the dominant follicle may abnormally persist as a follicular cyst. The fate of the first postpartum dominant follicle depends on luteinizing hormone (LH) pulse frequency, and failure to ovulate is usually a consequence of inadequate LH pulse frequency and reduced ovarian follicle estradiol, often associated with the metabolic stress of lactation and uterine bacterial infections (10, 12-14). Unfortunately, the return of ovarian cyclic activity is abnormal in about half of modern dairy cattle.

Uterine bacteria

Understanding of the microbes found in the postpartum genital tract has changed over the last 20 years. It was thought that the uterus was sterile during pregnancy, and became contaminated with non-specific bacteria from the animal and the environment after parturition. However, there is evidence that the uterus is not sterile and that specific microbes are adapted to the endometrium. Fluorescent probes to image bacteria and 16S ribosomal RNA sequencing of endometrium, provide evidence that there is a sparse microbiota in the uterus, even during pregnancy (15, 16). Bacteria identified in these studies include endometrial pathogens, such as *Trueperella pyogenes, Fusobacteria* species and *Prevotella* species. However, the uterine microbiota is substantially less abundant than in the gut or vagina, and the bacterial load is a fraction of that in postpartum uterine disease. Many bacteria in the postpartum uterus likely derive from the vagina, skin, gut and the environment. However, a bloom in the growth of pathogenic bacteria from the uterine microbiota after parturition may also help establish disease.

Postpartum uterine disease is polymicrobial. The microbial community in the uterus fluctuates during the postpartum period, with cycles of infection, elimination, and re-infection with bacteria. The bacteria most commonly cultured from animals with uterine disease are *Escherichia coli, T. pyogenes, Fusobacterium necrophorum*, and *Prevotella* and *Bacteroides* species (14, 17). Metagenomic techniques have found associations between uterine disease and *Bacteroidetes, Fusobacteria, Bacteroides, Proteobacteria* and *Firmicutes*, which are not readily cultured using standard techniques (18-22). Some of the metagenomic studies also find *E. coli* and *T. pyogenes* associated with disease, but others do not. Although the bacterial populations vary amongst animals, between diseases, and with time post partum, some bacteria are associated with uterine health, such as *Peptostreptococcus* and *Propionibacterium*. However, there remains a gap in understanding about which bacteria contribute to the pathogenesis of uterine disease. Taken together, the evidence is that *E. coli*, *T. pyogenes* and anaerobic bacteria are probably the main pathogens causing the clinical signs of uterine disease, but that other pathogens may initiate or contribute to endometrial pathology. There is evidence that specific co-infections foster disease. For example, *T. pyogenes, F. necrophorum* and *Prevotella* act synergistically to increase the likelihood of disease and the severity of endometritis (23, 24).

Novel strains of *E. coli* have been isolated from the uterus of animals with uterine disease (25, 26). These endometrial pathogenic *E. coli* (EnPEC) are more than twice as adherent and invasive for endometrial stromal cells than *E. coli* isolated from the uterus of clinically unaffected animals (25). In addition, EnPEC stimulate endometrial inflammation, and EnPEC establish disease in animal models. Lipopolysaccharide (LPS, endotoxin) and Type 1 fibrin D-mannose specific adhesion (commonly known as FimH) are important EnPEC virulence factors. Lipopolysaccharide is a major component of the outer membrane of Gram-negative bacteria, and provokes a robust inflammatory response when detected in animal tissues (27). Fimbria allow bacteria to adhere to host cells, and EnPEC FimH adhesion to endometrial cells was reduced by D-Mannose (25).

Trueperella pyogenes is the pathogen most associated with the severity of endometrial pathology, clinical disease, and in fertility (28-30). The link between *T. pyogenes* and disease probably depends on the virulence factor pyolysin. Pyolysin is a cholesterol-dependent cytolysin secreted by the bacterium, which binds cholesterol-rich domains in the plasma membrane of host cells to form pores, causing cell death by osmotic shock. Endometrial stromal cells are particularly sensitive to pyolysin, compared with endometrial epithelial cells or immune cells (31, 32). The stromal cytolysis caused by pyolysin may explain how *T. pyogenes* switches from a commensal in the uterus when the epithelium is intact, to causing uterine pathology once the epithelium is breached after parturition, allowing bacteria to reach stroma cells. The importance of *E. coli* and *T. pyogenes* for endometritis is supported by the ability to create models of endometritis by infusing *E. coli* and *T. pyogenes* into the uterus of naïve cattle (31, 33). However, it is notable that establishing an animal model of endometritis is fostered by supplying exogenous progesterone, which may suppress immune defenses and by debriding the endometrium immediately prior to infusion of bacteria, which disrupts the protective epithelium, allowing the pathogens to adhere to the tissues and invade the underlying stroma.

POSTPARTUM UTERINE DISEASE

The development of disease depends on the virulence of the pathogens and the ability of the pathogens to overcome an animal's tolerance and resistance. Many factors influence this balance, including genetics and the environment. The development of postpartum uterine disease in cows is particularly facilitated by several environmental risk factors.

Risk factors for uterine disease

Uterine disease is most common in *Bos taurus* dairy breeds that have high milk yields, and genetic selection for milk yield is often associated with the increased incidence of uterine disease after parturition. There is an opportunity to select animals for uterine health, and the heritability of metritis ranges from 0.08 to 0.26 (34, 35). However, environmental risk factors may be more important than genetic factors. For example, although some polymorphisms in genes associated with immunity have small effects on uterine health, environmental factors such as dystocia, parity, and ketosis are more predictive for uterine disease than the genetic markers (36).

The risk factors for postpartum uterine disease can be classified into three groups: trauma to the female genital tract, disorders of metabolism, or problems with hygiene. Trauma to the female genital tract is associated with retained placenta, dystocia, a large male calf, stillbirths, twins, first parity, and induction of parturition (37-40). Amongst these risk factors,

retained placenta has the strongest association with disease; for example, the odds ratio is > 40 for retained placenta causing clinical endometritis (40). Trauma is important in the development of endometritis, as trauma delays uterine involution to limit the physical expulsion of bacteria, and damages the endometrial epithelium to allow bacteria to reach the stroma.

Dairy cows are under metabolic stress because they cannot consume enough food to provide the metabolisable energy required for lactation. Typical cows producing 40 liters of milk/day require 200 MJ/day, which is three times the energy requirement for resting metabolism. Consequently, postpartum dairy cows catabolize tissues, develop insulin resistance, and have reduced blood concentrations of insulin-like growth factor (IGF-1), glucose and glutamine, and increased concentrations of ketones (41-43). One mechanism underlying the increased risk of disease is that metabolic stress compromises immune cell function (44, 45). Neutrophils have reduced superoxide production, impaired chemotaxis, and lower intracellular PMN glycogen in cows that develop metritis than healthy cows (44, 46). Neutrophils from cows with metritis also had about a third of the myeloperoxidase activity and cytochrome *c* reduction, compared with neutrophils from cows with normal uterine health around the time of parturition (45). Impaired neutrophil function is also evident before parturition, which may be important because cows that have stronger initial recruitment of inflammatory cells into the uterus have a more rapid resolution of uterine inflammation post partum (30).

Deficits in glucose or glutamine, which are the fundamental nutrients used by cells for energy, also reduce endometrial inflammatory responses (47, 48). Limiting the availability of glucose to ex vivo organ cultures of endometrium challenged with LPS or bacterial lipopeptides reduces the secretion of inflammatory mediators, such as the cytokines interleukin (IL)-1β and IL-6, and the chemokine IL-8 (also known as chemokine (C-X-C motif) ligand 8, CXCL8). (47). Similarly, depletion of glutamine, in the presence of abundant glucose, reduced the IL-1β, IL-6 and IL-8 response to LPS by at least 50% (48). The principal regulator of cellular energy is AMP-activated protein kinase (AMPK), which senses the ratio of AMP to ATP in the cytosol, and a homeostatic level of AMPK activation fosters optimal inflammatory responses (47). Cows also mobilize adipose tissue to satisfy the negative energy balance of lactation, increasing the peripheral plasma concentrations of non-esterified fatty acids (49, 50). These fatty acids are metabolized in tissues to acetyl coenzyme A to provide additional cellular energy; although, excess fatty acid oxidation leads to increased production of ketones. During an immune response, tissue cells tend to further exploit fatty acid oxidation to supply nutrients, whereas immune cells exposed to pathogens often increase fatty acid synthesis as part of their inflammatory response (51).

Whilst it is obvious that bacteria may reach the uterus from the environment, surprisingly, the cleanliness of the environment of the animals is not a dominant risk factor for postpartum uterine disease (40, 52). Rather than the presence of pathogens, development and progression of disease may depend more on risk factors that impair tolerance and immunity to the pathogens. Furthermore, which risk factors are important amongst trauma, metabolic stress, and hygiene depends on the type of uterine disease.

Definitions of uterine disease

Uterine disease is diagnosed by veterinarians on the basis of clinical signs. Definitions for the diagnosis of disease are established, and whilst there are limitations of clinical

definitions, it is important that veterinarians and researchers use a consistent approach to the diagnosis of disease (1, 53, 54).

Metritis

Metritis is most common within 10 days of parturition, and is characterized by an enlarged uterus, with a watery red-brown fluid to viscous off-white purulent uterine discharge (Figure 1A), which often has a fetid odor (53). The incidence of metritis varies between breed, country, and herd, but in a study of records from 97,318 cows in the USA, the lactation incidence of metritis, including retained placenta, was 21% (35). The severity of disease can be categorized by the signs of health:

- Grade 1 metritis. Animals with an abnormally enlarged uterus and a purulent uterine discharge, but without any systemic signs of ill health.
- Grade 2 metritis. Animals with an abnormally enlarged uterus and a purulent uterine discharge, with additional signs of systemic illness such as decreased milk yield, dullness, and fever.
- Grade 3 metritis; also called puerperal metritis or toxic metritis. Animals with an abnormally enlarged uterus and a purulent uterine discharge, with signs of toxemia, such as reduced appetite, cold extremities, and depression.

Metritis warrants treatment because it is painful and causes infertility (55, 56). In a meta-analysis of records from > 10,000 animals, metritis increased the time to first insemination by 7.2 days, reduced conception rates to first insemination by 20%, and increased the calving to conception interval by 18.6 days (56). Animals with grade 2 or 3 metritis are treated with parenteral broad-spectrum antibiotics for 3 to 5 days. However, a review of 17 studies using the third generation cephalosporin, ceftiofur, to treat metritis found that, whilst seven studies reported clinical improvement, there was no significant improvement in reproductive performance of the dairy herds (57). In our experience it is equally important to provide adequate nutrition, nonsteroidal anti-inflammatory drugs, and fluid therapy, if necessary. Of course antibiotics are directed at killing pathogens (resistance), whilst supportive therapy aims to limit tissue damage and return the animal to homoeostasis (tolerance).

Clinical endometritis

Clinical endometritis is defined as the presence of a purulent or mucopurulent uterine discharge detectable in the vagina of cattle 21 days or more postpartum. Examination of the vagina for purulent discharges with a clean gloved hand is simple, cheap and effective, with little risk of microbial contamination of the uterus in dairy cattle (14). The presence of pus in the vagina can also be detected using a vaginal speculum, or the Metricheck device comprising a rubber diaphragm on the end of a stainless steel rod. A 4-point grading system, based on the amount of pus in the vaginal mucus, is readily used to evaluate the severity of clinical endometritis, and is prognostic for the outcome of treatment (1, 58). The endometritis grade correlates with the presence of pathogenic organisms associated with uterine disease, but not the presence of non-pathogenic bacteria. However, evaluation of uterine disease is subjective and there is inter-operator and intra-operator variation (54, 59). There is also evidence that in some animals a purulent vaginal discharge associated with cervicitis or vaginitis impairs fertility, independent of endometritis (60). Irrespective of the exact pathology, these purulent conditions reflect an imbalance amongst pathogen virulence, host tolerance, and resistance.

The incidence of clinical endometritis is around 10 to 20%, with variation between breed, country and herd. In Canada, 16.9% of 1,865 dairy cows were affected (61); and, a survey of 19,870 Holstein cows in Germany found a lactation incidence of 19.2% for endometritis (62). Clinical endometritis is important because even after resolution of the disease, a history of endometritis increases the interval to first insemination by 11 days, and delays conception by 32 days, compared with animals that did not have endometritis (63). Cows with clinical endometritis between 20 and 33 days post partum are also 1.7 times more likely to be culled for reproductive failure, than cows without endometritis (61). The main treatments for endometritis are induction of estrus with prostaglandin $F_{2\alpha}$ in animals that have a corpus luteum, and intrauterine infusion of antimicrobial agents (58, 64). However, the treatment of endometritis attracts much discussion because there is 33% to 46% spontaneous 'self-cure', and because animals are sub-fertile even after treatment (63, 65, 66).

Subclinical endometritis

Subclinical endometritis is characterized by inflammation of the endometrium, in the absence of clinical signs of endometritis. The importance of subclinical endometritis emerged when cytological evidence of endometritis was associated with reduced fertility (67, 68). The cause of subclinical endometritis is not yet clear, and may include resolving bacterial infections, immuno-pathology, or tissue repair. Around twice as many animals with metritis subsequently develop subclinical endometritis, than animals without metritis (69). In the tissues of animals with subclinical endometritis there is increased expression of genes encoding inflammatory mediators such as the chemokines *CXCL5* and *CXCL8*, the cytokines *IL1A*, *IL1B* and *TNF*, and the acute phase protein *HP* (haptoglobin) (70-74). For example, 28-41 day post partum endometrial cytobrush samples from animals with subclinical endometritis, compared with normal animals, had 30-fold higher *IL6*, and > 50-fold higher *CXCL8* mRNA expression (71).

Subclinical endometritis is diagnosed when the proportion of neutrophils exceeds operator-defined thresholds, usually about 5% of cells, in samples collected by flushing the uterine lumen or by endometrial cytobrush 3 to 9 weeks post partum (53, 54, 75). There remain many open questions about subclinical endometritis because the etiology is not well established, and cytology does not always correlate with endometrial histopathology (29, 76). The proportion of animals affected also varies widely amongst studies and with different cellular cut-offs, with the incidence ranging from about 11% to > 40% of animals. A further issue is that there is no consensus about treatment for subclinical endometritis (75). In most diseases the acute response by neutrophils to infection is usually replaced by infiltration with macrophages, followed by resolution of inflammation (77, 78). So, the persistence of neutrophils in the endometrium 3 to 9 weeks post partum is unexpected and currently unexplained.

THE PATHOGENESIS OF UTERINE DISEASE

The increased incidence of uterine disease over the last 50 years coincides with intensification of dairy farming and breeding for increased milk yields to the extent that cows cannot consume enough food to meet the metabolic demand of producing > 35 L milk/day (42, 79). It is often argued that metabolic stress suppresses immunity, which leads to the development of postpartum uterine disease (42, 46, 49, 80). Our alternative view is that tolerance is more important than immunity, and that failure of the endometrium to tolerate

pathogens causes disease and leads to persistent inflammation, until tolerance and homeostasis are re-established.

We have use reaction norms to examine if postpartum uterine disease in cows is a failure of tolerance to pathogens and/or the ability of immunity to resist pathogens. Reaction norms are used in populations to compare the health status of organisms with their pathogen load in populations, allowing one to disentangle the relative contributions of resistance (Figure 1B) and tolerance (Figure 1C) (3). Data from a dairy herd of cows with similar genotype, where milk production, uterine health and bacterial load were recorded (14), were used to determine if milk yields in early lactation influenced tolerance or resistance to uterine pathogens. Postpartum cows under the metabolic stress of producing > 35 liters milk/day had worse endometrial health at the same uterine bacterial load as animals producing < 35 liters milk/day (Figure 1D), implying that metabolic stress perturbs uterine tolerance rather than immunity. Taken together, these findings, along with the risk factors associated with uterine disease, and the varied polymicrobial infections causing uterine disease, support our concept that uterine disease is less about which pathogens are present, and more about a failure in tolerance to pathogens. However, it is important to realize that once pathogens overcome endometrial tolerance, immunity and inflammation shape the progression and resolution of uterine disease.

Tolerance

Tolerance is about damage limitation in the face of pathogens, and we propose several mechanisms for tolerance to uterine pathogens. The vulva, vagina, cervix, and the cervical mucus plug provide physical barriers to ascending infections during gestation, until they are breached during and after parturition. In humans, commensal *Lactobacilli* species in the vaginal microbiota provide additional protection against pathogens, probably by competing for nutrients and generating an acid environment (81). However, cattle have < 1% relative abundance of *Lactobacilli* in the vagina compared with 70% in women, and a less acid vaginal environment of pH 7 in cows compared with pH 4.5 for humans (82). However, infusion of *Lactobacilli* into the prepartum bovine vagina reduced the incidence of postpartum metritis to 15% compared with 38% in control animals (83).

The mucus layer on the apical surface of epithelia provides a substantial obstacle to microbes (Figure 2). The secretion and character of mucus in the genital tract is under the control of ovarian steroids, and during estrus mucus flow increases considerably. Interestingly, the expression of *MUC1* mRNA was increased 2-fold when endometrial epithelial cells were treated with LPS (84), and by a similar amount in endometrial cytobrush samples from cows with clinical endometritis (74). Lysozyme expression is also increased in the endometrium of cows with uterine inflammation, and lysozyme digests the peptidoglycans found in the cell walls of bacteria (85). Antimicrobial peptides and mucosal glycoproteins cover the mucosa of the vagina, cervix and endometrium, where they also neutralize bacteria and prevent them reaching the plasma membrane of the epithelia. The principal cysteine-rich, cationic, antimicrobial peptides expressed by bovine endometrial epithelium include β-defensins, lingual antimicrobial peptide (LAP), and tracheal antimicrobial peptide (TAP); several gene transcripts for antimicrobial peptides are more abundant in the face of microbial challenge, which links immunity and tolerance (84, 86).

The next line of endometrial defense is the columnar epithelium, which has tight junctions between cells to separate the apical and basolateral components of the endometrium

(Figure 2). Epithelial cells are at least twice as resistant than stromal cells to cytolysis caused by *T. pyogenes* or pyolysin (31). The greater tolerance of epithelial cells to pyolysin may be because they contain less cholesterol in their plasma membrane than stromal cells. About 90% of the cholesterol in cells is contained within the plasma membrane, and cholesterol concentrations are tightly regulated by cholesterol uptake, cholesterol efflux transporters, and by cholesterol synthesis (87). The first steps in cholesterol synthesis are encompassed by the mevalonate pathway, converting acetyl-CoA to isoprenoids, which are then converted to squalene, and ultimately to cholesterol. The ability of endometrial stromal cells to tolerate pyolysin can be increased using methyl-β-cyclodextrin to reduce cellular cholesterol, statins to inhibit the mevalonate pathway, squalene synthase inhibitors, or supplying exogenous isoprenoids (31, 32, 88, 89). For example, endometrial stromal cell viability increased from 14% in controls challenged with pyolysin to 34% in cells treated prior to pyolysin challenge with 10 μM zaragozic acid to inhibit squalene synthase (88). Infusing statins or squalene synthase inhibitors into the postpartum uterus might increase endometrial tolerance to pathogens, and limit the severity of uterine disease. Interestingly, statins or inhibition of squalene synthase also modulates innate immunity in the endometrium. In particular, inhibiting squalene synthase reduces the IL-6 and IL-8 inflammatory response to LPS by > 50% in endometrial epithelial and stromal cells (90).

The endometrial epithelium is often denuded during the postpartum period, which allows pathogens to reach the extracellular matrix. Damage control is an important aspect of tolerance, and there is evidence that endometritis affects extracellular matrix turnover. Interestingly, genes involved in extracellular matrix homeostasis, such as the matrix metallopeptidases genes *MMP1, MMP3, MMP9* and *MMP13*, are differentially regulated in cows that develop uterine disease when they have severe negative energy balance compared with animals that are in energy balance and do not develop disease (91). Furthermore, the cytokine TNFα increases the production of metallopeptidases MMP-2 and MMP-9 (92).

Whilst physical barriers and secreted proteins are important for tolerance to microbes in the female genital tract, there are some caveats. First, microbes have evolved counter-measures and strategies to avoid many host defenses. For example, some bacteria produce enzymes that lyse mucus to penetrate the protective layer, and many bacteria secrete proteases that can disrupt peptides and proteins produced by the host (93). Second, immunity is principally responsible for initiating rapid inflammatory responses to resist microbial infections if the tolerance mechanisms are overcome (5, 94).

Resistance

Resistance provides pathogen control, and principally depends on innate and adaptive immunity. Innate immunity provides immediate, non-specific defense against pathogens, and does not depend on prior exposure to microbes (27, 94). Adaptive immunity employs antigen-specific receptors and the response may take several days, depending on prior exposure to the antigen. Innate immunity appears to dominate resistance to pathogens in the endometrium. Cows with uterine disease have increased expression of genes in the endometrium that are typical of innate immunity, including: cytokines *IL1A, IL1B, IL6, TNF* and *IL12A*, cytokine receptors *IL1R1* and *IL1R2,* chemokines *CXCL5* and *CXCXL8*, prostaglandin synthesis enzymes *PTGS1, PTGS2, PTGDS* and *PGES,* antimicrobial peptides *LAP*, *TAP, DEFB5* and *DEFB1*, and acute phase proteins *HP* and *SAA3* (70-74, 86, 95, 96). Cows with clinical endometritis also have higher concentrations of IL-1α, IL-1β

and IL-6 in uterine fluid than healthy cows (97, 98). The influx of neutrophils into the uterus is also typical of innate immunity, and this rapid and robust response is important for postpartum uterine health (30).

Innate immunity

Innate immunity encompasses multiple host resistance mechanisms ranging from acute phase proteins to cytokines (27). Acute phase proteins are synthesized in the liver, in response to increased peripheral plasma concentrations of cytokines such as IL-6 (99). Acute phase proteins help restore homeostasis after infection or tissue damage, with roles in hemostasis typified by the action of fibrinogen, anti-microbial effects, and attracting and activating phagocytes. The peripheral plasma concentrations of acute phase proteins, such as α_1-acid glycoprotein, haptoglobin and ceruloplasmin, are associated with the severity of bacterial contamination in the postpartum uterus, particularly the presence of *E. coli* and *T. pyogenes* (100, 101). However, the acute phase response is also initiated by trauma (99); and concentrations of α_1-acid glycoprotein, haptoglobin and ceruloplasmin increase with parturition**,** and then decline as uterine involution progresses (101). There is also local expression of genes encoding acute phase proteins in the uterus and ovary, which may provide further localized protection from pathogens (70, 86, 102). Antimicrobial molecules, such as S100 calgranulins and cathelicidin, are also more abundant in the endometrium of animals with uterine disease (91, 103, 104). These proteins are often abundant in neutrophils**,** and are associated with antimicrobial activity, attracting and activating immune cells.

Complement is also an important component of innate defense against microbial infections. The complement system comprises about 20 proteins, which generate lytic complexes at the surface of pathogens, and provide opsonins such as C1b and C3b, which interact with cell surface receptors to promote phagocytosis by neutrophils and macrophages. Components of the complement system, such as the genes *C1QA*, *C1QB*, *C1QC*, *C3* and *C8*, are differentially expressed in the endometrium of diseased postpartum cows with more severe negative energy balance (91).

Much of the innate immune response depends on the detection of microbes by host cells. Innate immunity is founded on the discovery that eukaryotic cells possess pattern recognition receptors that bind pathogen-associated molecular patterns (PAMPs) (105, 106). These PAMPs are usually highly conserved molecules found in prokaryotes but not eukaryotes, and include LPS, lipopeptides, flagellin, and microbial RNA and DNA. Receptors such as Toll-like Receptors (TLRs) and NOD-like Receptors (NLRs) bind PAMPs (27, 94). For example, TLR4 binds to LPS, TLR2/TLR1 and TLR2/TLR6 dimers bind bacterial lipopeptides, and TLR5 binds flagellin. Binding of PAMPs to TLRs activates NF-κB and MAPK intracellular signaling pathways, which result in the production of antimicrobial peptides, such as β-defensins, LAP and TAP, and inflammatory mediators, such as IL-1β, IL-6, IL-8 and prostaglandin E_2 (Figure 3). Intracellular pattern recognition receptors, such as NLRP3 (nucleotide-binding domain and leucine rich repeat pyrin 3 domain), principally detect microbes that invade cells, activating the multiprotein inflammasome complex, leading to caspase-1 cleavage of pro-IL-1β to mature IL-1β protein (107). Inflammatory mediators attract and/or activate immune cells to clear bacteria (27, 94). Cytokines bind their cognate receptors, leading to inflammation and further production of antimicrobial peptides, eicosanoids and reactive oxygen species. Cytokines in the peripheral plasma also drive systemic inflammatory responses, including pyrexia, generalized vasodilation, and release of

acute phase proteins from hepatocytes. Chemokines, such as IL-8, attract neutrophils and monocytes to the site of infection to engulf and kill bacteria.

Innate immunity in the endometrium

Pattern recognition receptors are principally expressed by hematopoietic cells such as neutrophils, macrophages, and dendritic cells (27, 94). However, bovine endometrial epithelial and stromal cells also express most *TLR* genes (84). These epithelial and stromal cells respond to bacteria, LPS, and lipopeptides, via TLR1, TLR2, TLR4 and TLR6, activating the NF-κB and MAPK pathways, which stimulate the production of inflammatory mediators, including IL-1β, IL-6, IL-8 and prostaglandin E_2 (108-110). In addition, antimicrobial peptide *LAP* and *TAP* gene transcription is increased by > 100 fold when epithelial cells are stimulated with LPS (84). The polarized epithelial cells also spatially direct some of their inflammatory responses (Figure 3), with apical challenge using LPS stimulating predominantly apical secretion of IL-8, and basolateral secretion of prostaglandins (111). More striking is that apical or basolateral challenge of epithelial cells with LPS, always directs IL-6 secretion apically (112). These spatial responses may direct immune cells to the site of infection, regulate the immune response, or be associated with physiological roles for some of these inflammatory mediators. For example, LPS switches epithelial cell secretion from prostaglandin $F_{2\alpha}$ to prostaglandin E_2, and this is not reversed by administering oxytocin to mimic the luteolytic signal (113). This prostaglandin switch has important implications for fertility, as LPS may counter the physiological production of prostaglandin $F_{2\alpha}$ from epithelial cells for luteolysis.

Excessive inflammation leads to immuno-pathology or septic shock, and so a series of checks and balances are in place to scale inflammation to meet the level of microbial threat during the progression of an infection, and to resolve inflammation after infections are cleared (114). One example in the bovine endometrium is a positive feedback role for the IL-6 receptor (IL6R) and signal transducer and activator of transcription-3 (STAT3) (115). Stromal cell IL6R binds the IL-6 released during the initial stage of infection, leading to activation of STAT3, which in turn stimulates further increases in the secretion of IL-6 and IL-8 (Figure 3).

Damage-associated molecular patterns (DAMPs) are also used to modulate the intensity of the inflammatory response initiated by PAMPs (114). Pattern recognition receptors bind DAMPs to help cells sense danger (116, 117). Dying or damaged mammalian cells release DAMPs such as HMGB1, IL-1α, mitochondrial DNA and ATP (116). Endometrial epithelial and stromal cells employ IL-1α as the principal DAMP (97). Cells accumulate IL-1α intracellularly in response to LPS, and IL-1α is only released when cells are damaged (Figure 3). This may be important for *T. pyogenes* infections where pyolysin forms pores in the plasma membrane of endometrial cells (31, 32, 88). The IL-1α then binds to the cognate receptor, IL-1R, to stimulate other cells to release more inflammatory mediators, such as IL-6. Interestingly, innate immune responses are also activated by pore-forming toxins that induce ion fluxes across the plasma membrane (118, 119). However, whilst *T. pyogenes* stimulate inflammatory responses, pyolysin did not stimulate inflammation in bovine endometrial or immune cells (31).

Innate immunity is an evolutionary ancient system, and so it is not surprising that it is integrated with other cellular homeostatic and metabolic pathways (78). Cows with a severe negative energy balance have persistent uterine inflammation, whereas animals with mild

negative energy balance recover their energy balance and repair their endometrium by two weeks after parturition (91). There is even evidence that reduced appetite prior to parturition increases the risk of postpartum uterine disease (38). The response to pathogen molecules is also energetically expensive (120). A striking example is that animals use > 1 kg of glucose in the first 12 hours after challenge with LPS (120). Endometrial tissue exposed to LPS in vitro also has an increased demand for glucose (47). Metabolic stress compromising innate immunity may result in inefficient clearance of bacteria from the endometrium.

Adaptive immunity

Adaptive immunity in the endometrium is also of interest for endometritis, but there remain gaps in knowledge. Early work identified areas rich in T cells and B cells in the postpartum endometrium, often as lymphocytic foci within the stroma (9, 28). Adaptive immune responses are also evident in postpartum animals, with increased abundance of antibodies (121). Increased levels of circulating antibodies are associated with less postpartum uterine disease (122). However, the importance of adaptive immunity in countering uterine infections is unclear as preliminary data on vaccines for metritis containing components of *E. coli*, *F. necrophorum* and/or *T. pyogenes* suggests that vaccines can provide some protection against disease (123, 124), but other studies do not support this concept (125). Furthermore, postpartum endometritis often occurs after successive calvings, and adaptive immunity does not appear to provide long-term protection.

IMPACT OF UTERINE DISEASE ON OVARIAN FUNCTION

Whilst failures in damage limitation and pathogen control shape uterine disease, the ovary was previously considered to be tolerant to infections. However, links between the postpartum uterus and ovary were proposed because the first dominant follicle after parturition is twice as likely to be in the ovary contralateral to the previously gravid uterine horn (126, 127). The role of uterine disease in this link between uterus and ovary was highlighted by observations that dairy cows with uterine infections have slower growth of the first postpartum dominant follicle from 7 to 16 days after parturition, lower peripheral plasma estradiol concentrations, and are less likely to ovulate (14). In a subsequent study, not only was follicle growth and estradiol secretion reduced in cows with uterine infections, but also there were lower circulating concentrations of progesterone after formation of the first postpartum corpus luteum (128). Subclinical endometritis 21 days postpartum did not affect follicle growth, but even 9 weeks after parturition intra-follicular estradiol concentrations were lower in cows with uterine infection compared with normal cows (129).

There is an intimate vascular connection between the uterus and ovary, with the venous drainage from the uterus an important route for prostaglandin $F_{2\alpha}$ reaching the ovarian artery to initiate luteolysis (130). This vasculature is markedly enlarged in the postpartum period, and so PAMPs and inflammatory mediators may also use this local route to reach the ovary (Figure 4). The concentration of LPS in follicular fluid aspirated from dominant follicles in vivo is correlated with the severity of uterine disease (131). In follicles collected after slaughter, follicles that contained higher concentrations of LPS had lower concentrations of estradiol, and lower expression of mRNA for the steroidogenic enzymes *CYP17A1* and *CYP19A1* (132). Follicular fluid LPS concentrations are also higher in follicles of cows that did not ovulate the first postpartum follicle compared with cows that ovulated (133).

Little is known about tolerance and resistance in the ovary. The physical protection provided to the oocyte by the ovarian follicle and the zona pellucida, and the suspended development of primordial follicles and meiotic arrest, are concordant with the concept of tolerance. However, healthy ovarian follicles do not contain hematopoietic immune cells, so ovarian follicle resistance must rely on the granulosa cells or oocyte (131, 134). Granulosa cells isolated from growing or dominant ovarian follicles express *TLR1-10* mRNA, and LPS or bacterial lipopeptides stimulate the secretion of IL-1β, IL-6, CXCL1, CXCL2, CXCL3 and IL-8 protein from granulosa cells (134-136). The functional role for TLR4 and TLR2 in granulosa cells was confirmed using siRNA to deplete gene expression and by inhibiting the MAPK signaling pathway (134, 135). The abundance of IL-6 secreted by granulosa cells from beef or dairy animals is similar to that for macrophage response to LPS (134). Lipopolysaccharide also limits granulosa cell estradiol production by reducing the expression of *CYP17A1* and *CYP19A1*, and reducing aromatase protein levels (131, 132, 135). Whilst there are direct effects of LPS on granulosa cell function, there may also be indirect effects because IL-6, IL-8 and TNFα perturb granulosa cell steroidogenesis in vitro (137-139). In addition, 2-day treatment with 100 ng/ml IL-6 perturbed the proliferation of granulosa cells from emerging and dominant follicles, with 32% and 55% fewer cells than controls, respectively (137). The impact of uterine infection may also affect even the earliest stages of follicle development. In ex vivo experiments, LPS reduced the primordial ovarian follicle pool, with an associated increase in primordial follicle activation, and loss of primordial follicle regulatory proteins (140).

Resistance mechanisms are evident in cumulus-oocyte complexes as LPS also stimulates the secretion of IL-6 (134). The LPS also activates cumulus expansion in vitro, and inappropriate timing of cumulus expansion may contribute to infertility, because expansion is closely coordinated with ovulation. Furthermore, LPS or IL-6 might reach the oocyte via the cytoplasmic trans-zonal projections from granulosa cells that synapse on the oolema. Indeed, LPS increases the incidence of meiotic arrest and germinal vesicle breakdown failure in bovine oocytes (134). Treatment of cumulus-oocyte complexes with LPS or a bacterial lipoprotein also perturbed the expression of *GDF9* and *NLRP5*, which are involved in oocyte maturation (141). The effects of infection on fertility are not only evident during disease, but also for some time after because oocyte development takes about 120 days, from the primordial follicle stage to ovulation of a cumulus-oocyte complex. Thus, in cows inseminated 60 to 120 days after parturition, the oocytes that are ovulated will have started their development during the early postpartum period when they may have been exposed to uterine disease. This effect is similar to how greater body condition loss from 3 to 5 weeks post partum reduces conception rate several weeks later (2, 142).

Another mechanism linking uterine disease to ovarian dysfunction is that PAMPs and cytokines perturb the function of the hypothalamus and the pituitary, reducing the release of gonadotrophin-releasing hormone (GnRH) and luteinizing hormone (LH), which regulate ovarian function (13, 143). In postpartum cows, LPS in the uterus suppressed the LH surge, and prevented ovulation (13). In addition, during the follicular phase LPS decreases LH pulse frequency, decreases estradiol and interrupts the LH surge and ovulation in cattle (144, 145). However, the peripheral plasma concentrations of FSH are unaffected by uterine disease in cattle, so that recurrent waves of follicles develop in the ovary, even during uterine disease (14).

One consideration about the links between the uterus and ovary is that ovarian steroid hormones can also influence the development of postpartum uterine disease. Progesterone

is immuno-suppressive, whilst estradiol may enhance immunity and was used to treat uterine disease (146). However, there are conflicting data about how estradiol could increase uterine resistance. Exogenous estradiol infused into the bovine uterus during the postpartum period increased the abundance of bacteria in the uterus (147). Furthermore, the stage of the estrous cycle, or exogenous progesterone or estradiol, did not modulate innate immunity in ex vivo organ cultures of bovine endometrium (148). Similarly, treatment with estradiol or progesterone, or inhibitors of estradiol or progesterone nuclear receptors, did not affect IL-1β, IL-6 or IL-8 protein or *IL1B*, *IL6*, *CXCL8* or *CCL5* gene expression by endometrial cells or macrophages (148). More work is needed on how ovarian steroids affect the resilience of the uterus to pathogens.

CONCLUSIONS

Whilst there is a clear understanding of the clinical aspects and implication of postpartum uterine disease, and some of the mechanisms of pathology, there are important outstanding questions. The most obvious question is why are modern, high-milk-yield cows so susceptible to metritis and endometritis? Allied to this, is what can be done to prevent uterine disease? Answering these questions is vital for sustainable intensification of the dairy industry over the next 50 years (2). Genetic selection for more resilient animals that are less susceptible to uterine disease is a priority for the dairy industry. However, here we argue that the increasing incidence of postpartum uterine disease could be caused by failures in tolerance to bacteria in the endometrium. Once endometrial tolerance is overcome by the pathogens, then a prompt and robust innate immune response is essential to counter the pathogens. However, there is evidence that the metabolic stress associated with lactation compromises both tolerance and immunity. We propose that failures in endometrial tolerance to pathogenic bacteria and the subsequent innate immune response shape postpartum uterine disease.

Failures in tolerance would better explain why antibiotics used in animals with uterine disease to kill the pathogens are of little benefit to fertility. We suggest that prevention is better than cure for uterine disease, not only because the disease causes pain and suffering, but also because uterine infections perturb ovarian function and oocyte health. Developing new ways to counter postpartum uterine disease will come from improved understanding of tolerance and resistance in the postpartum genital tract. Perhaps the best advice is to optimize animal nutrition and management to increase the tolerance of parturient and postpartum animals, so that they are better able to limit the impact of uterine pathogens, without developing uterine disease.

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REFERENCE ANNOTATIONS

3. Raberg et al: Provides a framework for exploring resistance and tolerance in animals.

15. Sheldon et al: Evidence for how postpartum uterine disease affects ovarian follicle growth and development in vivo.

14. Karstrup et al: Visualization of bacteria in the endometrium of animals during pregnancy showing that the uterus is not sterile.

31. Amos: Dissection of the role of pyolysin in the bovine endometrium and identifying the sensitivity of stromal cells to pyolysin.

53. Sheldon et al: Sets out the definitions and methods for scoring postpartum uterine disease.

67. Kasimanickam et al: Recognition of the importance of subclinical endometritis in cattle.

105. Lemaitre et al: Discovery of a role for Toll in invertebrates, founding the field of innate immunity.

106. Poltorak et al : Discovery of a role for Tlr4 in recognition of LPS in mice, founding the field of innate immunity in mammals.

108. Herath et al: First evidence for the role of innate immunity in bovine endometrial cells.

131. Herath et al: Finding of LPS in ovarian follicular fluid, and recognition that bovine granulosa cells have roles in innate immunity.

SIDEBAR

ANIMAL RESILIENCE DEPENDS ON RESISTANCE AND TOLERANCE

The nomenclature for tolerance and resistance in animals is somewhat confused by semantics and scientific traditions because it originates from the field of plant ecology. The simplest approach is to consider an animal's resilience to infection as the combination of the animal's tolerance and resistance to the pathogens (3). Tolerance is the ability to limit the disease severity induced by a given pathogen burden (4-6). Resistance is the ability to limit the pathogen burden, and is usually the function of immunity. However, tolerance has also been described using words such as "resilience" and "endurance". Tolerance is often equated with damage control, whilst resistance largely depends on immunity for pathogen control. Whilst resistance mechanisms are widely studied, there is a scarcity of knowledge about the mechanisms of tolerance in animals. However, tolerance has the advantage over immunity in that tolerance helps prevent disease developing, whereas immunity is a response to the progression of infection. Unfortunately, resistance is often negatively correlated with tolerance (6). Thus, selecting for milk yield in the face of uterine disease may inadvertently reduce tolerance. In our view, prevention is better than cure, and tolerance in cows warrants further investigation to facilitate sustainable agriculture.

TERMS AND DEFINITIONS LIST

Acute phase protein: proteins that change serum concentration by > 25% in response to inflammatory cytokines, and help counter infections.

Chemokine: proteins that stimulate chemotaxis - movement of leukocytes toward sites of infection.

Cytokine: signaling molecules that mediate and regulate immunity, inflammation, and hematopoiesis.

Pathogen: an organism that causes disease in a host.

PAMPs: pathogen-associated molecular patterns are molecules found in prokaryotes that are recognized by the innate immune system in eukaryotes.

Reaction norm: a curve that relates, for a given genotype, the contribution of environmental variation to observed phenotypic variation.

Resistance is the ability to limit the pathogen burden, and is usually the function of immunity.

Sepsis: life-threatening organ dysfunction caused by a dysregulated host response to infection.

Tolerance is the ability to limit the disease severity induced by a given pathogen burden.

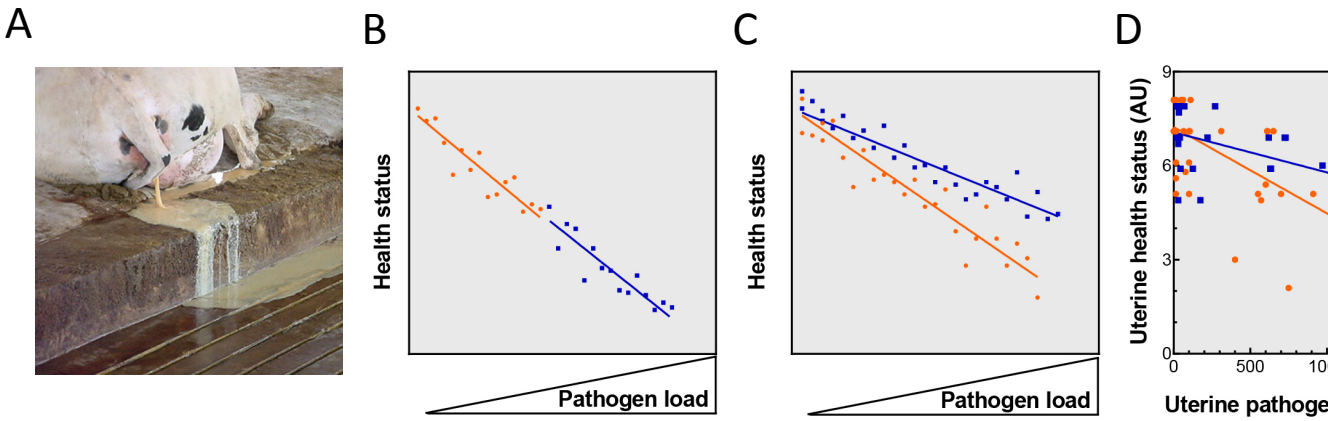
FIGURE LEGENDS

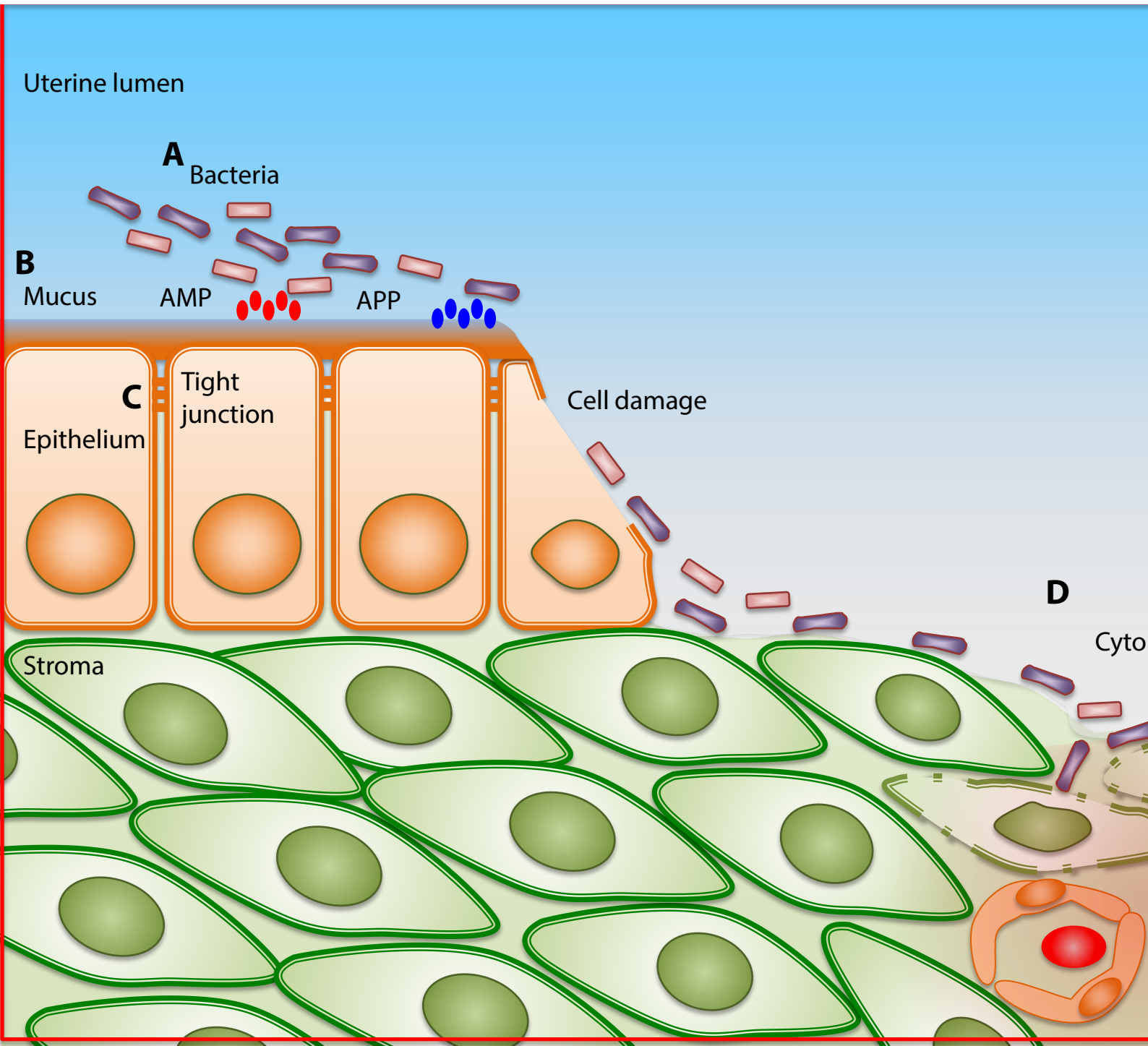
Figure 1. Uterine disease is associated with impaired tolerance. (A) Cows with postpartum uterine disease discharge pus from the uterus. (B) Schematic reaction norms of health status against pathogen load for two groups of animals with similar tolerance, where each animal is represented by a symbol (■ or ●), but the reaction norm for the blue group (▬) indicates that these animals have impaired immunity, with reduced health status and more pathogens, compared with the red group (▬); or, (C) two groups with similar immunity, but the reaction norm for the red group (▬) indicates that these animals are less tolerant, with reduced health at the same pathogen load as the blue group (▬); based on the concepts proposed by Raberg et al (3). (D) Using data from a previously published study of postpartum uterine clinical health score and uterine bacterial load for dairy cows producing > 35 L milk/day (●, n = 56) and animals producing < 35 L milk/day (■, n = 34) (14), the reaction norm for the cows producing > 35 L milk/day (▬) indicates that these metabolically-stressed animals have impaired tolerance, with reduced health at the same pathogen load as the animals producing < 35 L milk/day (▬).

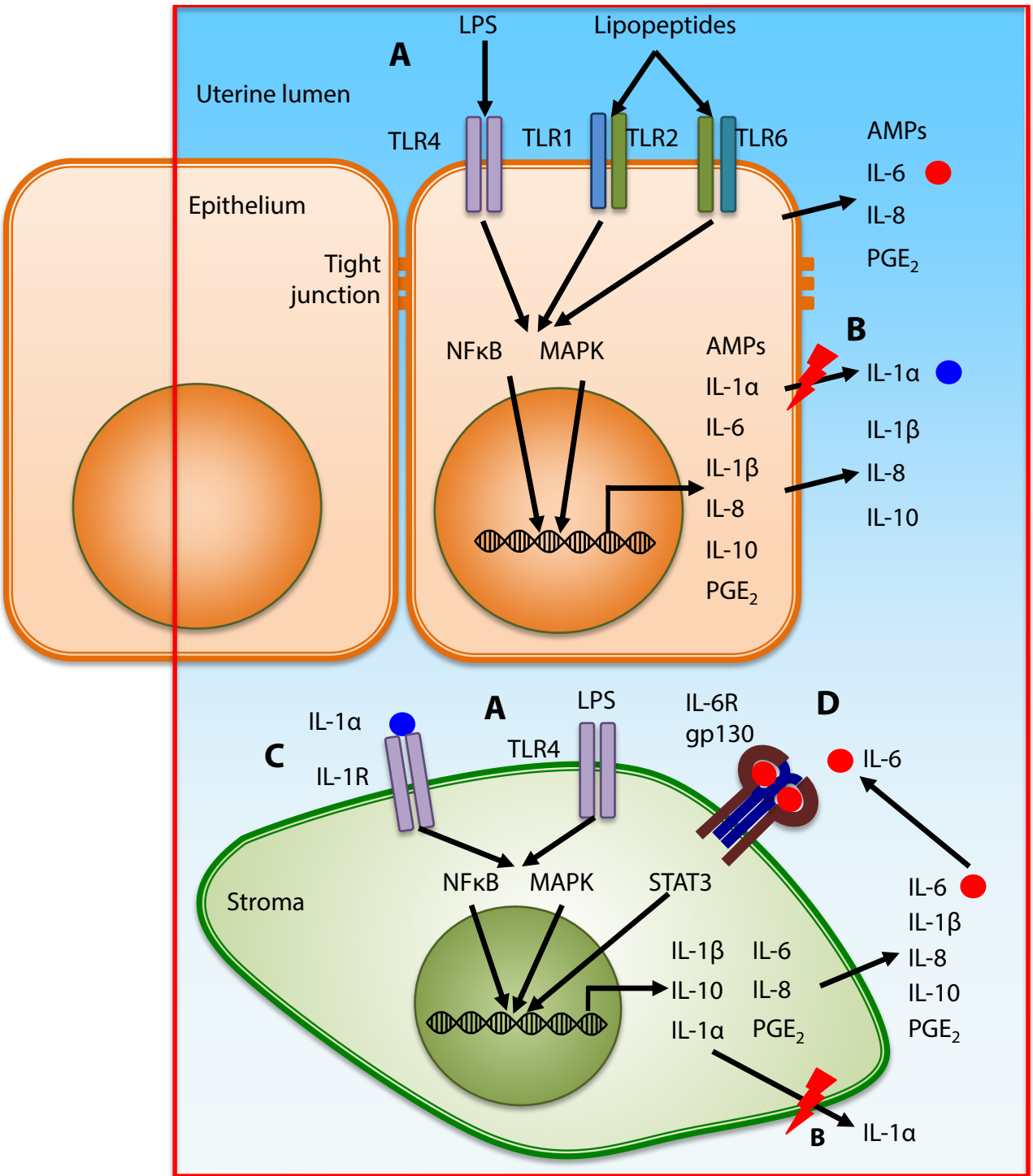
Figure 2. Development of postpartum endometritis. (A) After parturition there is a bloom of bacterial growth in the uterus, and multiple pathogens compete with commensal bacteria in the endometrium. (B) The mucus layer in the endometrium helps prevent bacteria reaching the epithelium, and antimicrobial peptides (AMP) and acute phase proteins (APP) neutralize bacteria and their virulence factors. (C) Tight junctions between epithelial cells prevent bacteria penetrating to the underlying stroma; although, after parturition epithelial cell damage exposes the stroma. (D) If bacteria reach the stroma they often cause cell damage and cytolysis, and provoke inflammatory responses, including the influx of neutrophils from the peripheral circulation.

Figure 3. The innate immune response in the endometrium. (A) Endometrial epithelial (orange) and stromal (green) cells express functional TLR4, and TLR2/TLR1 and TLR2/TLR6 heterodimers. Binding of PAMPs to TLRs activates the NFκB and MAPK signalling pathways, which lead to the transcription of genes that encode several inflammatory mediators, including antimicrobial peptides (AMPs), cytokines, chemokines and prostaglandins. Whilst most inflammatory mediators are released from cells into the extracellular fluid, in the polarized epithelial cells specific inflammatory mediators are secreted apically. (B) Cells release the intracellular cytokine IL-1α (●)if cells are also damaged (⚡). (C) In a paracrine manner, IL-1α can bind to the IL-1R of nearby cells, further activating the NFκB and MAPK signaling pathways. (D) Epithelial and stromal cells respond differently to IL-6 (●), and in stromal cells IL-6 has a positive feedback through the IL-6R/gp130 receptor heterodimer and STAT3 signaling to enhance the secretion of IL-6 and IL-8.

Figure 4. Uterine disease affects ovarian function. (A) Pathogen-associated molecular patterns (PAMPs), such as LPS, and inflammatory mediators, such as cytokines and prostaglandins, are abundant in the uterus of animals with postpartum endometritis. (B) The PAMPs and inflammatory mediators reach the vasculature via the uterine vein, and (C) then may reach the ovary by counter-current mechanisms with the ovarian arterial circulation, local lymphatics, or the peripheral circulation. (D) In the ovary PAMPs and inflammatory meditators perturb ovarian follicle development and oocyte health.


A
B
C
D
Health status
Pathogen load
Health status
Pathogen load
Uterine health status (AU)
9
6
3
0
0
500
Uterine pathoge


Uterine lumen
A
Bacteria
B
Mucus
AMP
APP
C
Epithelium
Tight
junction
Cell damage
D
Cyto
Stroma


LPS
Lipopeptides
A
Uterine lumen
TLR4
TLR1
TLR2
TLR6
AMPs
IL-6
IL-8
PGE2
Epithelium
Tight
junction
NFκB
MAPK
B
AMPs
IL-1α
IL-6
IL-1β
IL-8
IL-10
PGE2
IL-1α
IL-1β
IL-8
IL-10
IL-1α
IL-1R
C
A
LPS
TLR4
IL-6R
gp130
D
IL-6
Stroma
NFκB
MAPK
STAT3
IL-1β
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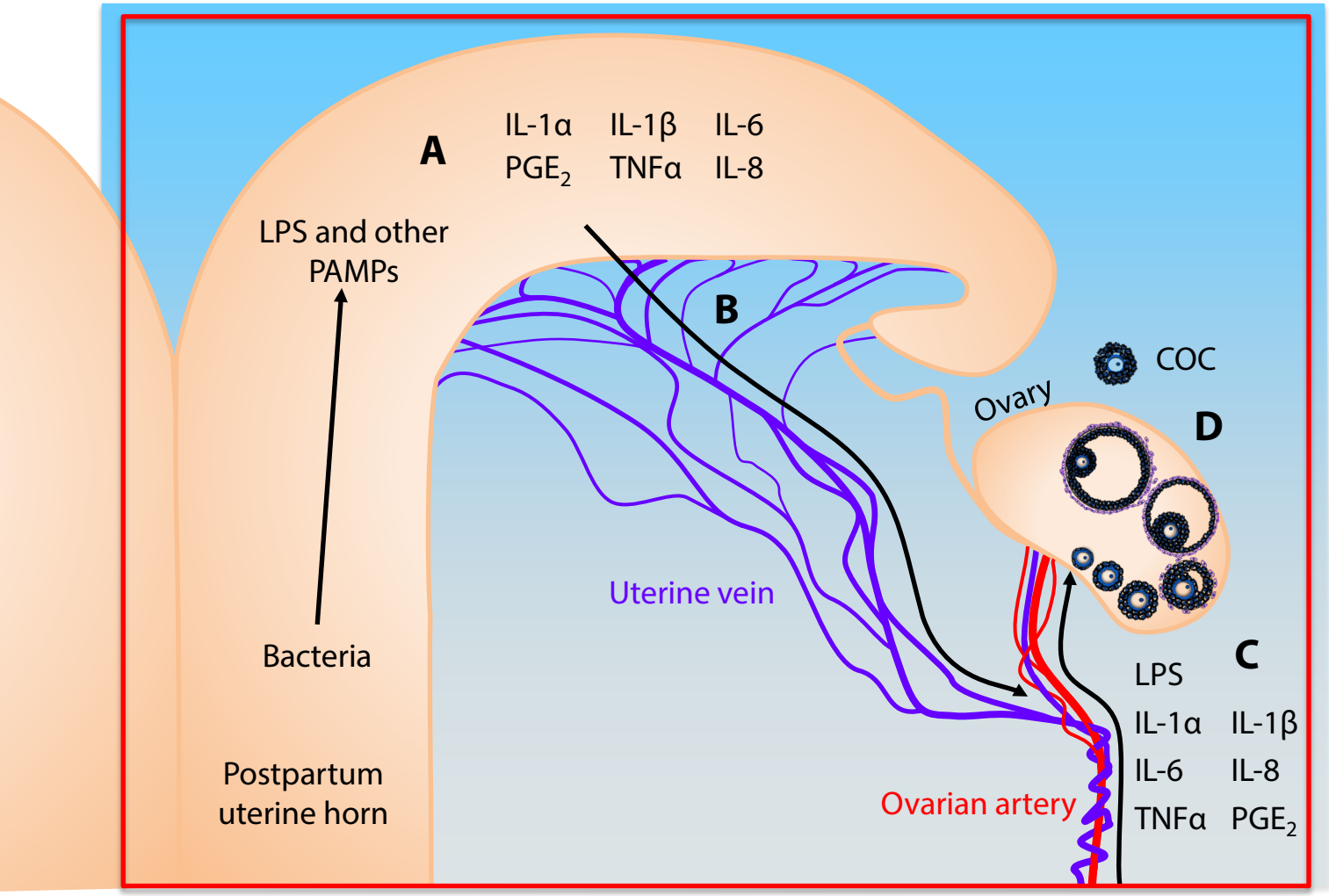
IL-1β
IL-8
IL-10
PGE2
B
IL-1α


A
IL-1α IL-1β IL-6
PGE2 TNFα IL-8
LPS and other
PAMPs
B
COC
Ovary
D
Uterine vein
Bacteria
C
LPS
IL-1α IL-1β
IL-6 IL-8
TNFα PGE2
Ovarian artery
Postpartum
uterine horn