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PHYSIOLOGY AND ENDOCRINOLOGY SYMPOSIUM: Uterine infection: Linking infection and innate immunity with infertility in the high-producing dairy cow[1,2]

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ABSTRACT: Uterine contamination with bacteria is ubiquitous in the postpartum dairy cow. Nearly one-half of all postpartum dairy cows develop clinical disease resulting in metritis and endometritis, which cause depressed milk production and infertility. The causative links between uterine infection and infertility include a hostile uterine environment, disrupted endocrine signaling, and perturbations in ovarian function and oocyte development. In this review we consider the various mechanisms linking uterine infection with infertility in the dairy cow, specifically 1) innate immune signaling in the endometrium, 2) alteration in endocrine signaling in response to infectious agents, and 3) impacts of infection on ovarian function, oocyte development, and follicular development. Normal ovarian follicular and oocyte development requires a series of temporally and spatially orchestrated events; however, several of the cellular pathways required for ovarian function are also used during the innate immune response to bacterial pathogens. We propose that activation of cellular pathways during this immune response has a negative impact on ovarian physiology, which is manifest as infertility detected after the clearance of the bacteria. This review highlights how new insights into infection and immunity in cattle are linked to infertility.

Key words: cow, immunity, infection, oocyte, ovary, uterus

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INTRODUCTION

The uterine mucosal environment is protected from pathogenic bacterial infiltration by physical anatomical barriers and active molecular mechanisms. However, in comparison to non-dairy cattle breeds, high-milk-yield dairy breeds, such as the Holstein-Friesian, are prone to uterine bacterial contamination when these mechanisms fail, primarily following parturition. In the dairy cow, parturition generates considerable tissue damage to the endometrium and cervix, damage which results in the failure of the anatomical barriers to prevent ascending uterine bacterial infiltration. Although bacterial infections are normally cleared within 3 to 5 wk after parturition, many cows display signs of impaired fertility, including reduced conception, lower submission rates (i.e., percentage of cows inseminated within 28 d from the start of the mating period), and increased calving-to-conception intervals, and these signs occur temporally after the resolution of the signs of uterine disease (Borsberry and Dobson, 1989; McDougall, 2001; LeBlanc et al., 2002). The innate immune response to bacteria is key to rapidly clear-

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ing infection (Herath et al., 2006; Davies et al., 2008; Cronin et al., 2012; Turner et al., 2014). Recruitment of hematopoietic immune cells and the inflammatory response, including secretion of cytokine and chemokines, all combine to clear the bacterial infection and restore hemostatic function of the endometrium (Sheldon and Roberts, 2010). However, evidence is emerging that these inflammatory events have long-term consequences on the fertility of dairy cows by negatively impacting endocrine signaling, uterine homeostasis, and ovarian function.

POSTPARTUM DISEASE IN THE DAIRY COW

Under normal circumstances, the anatomical barrier of the cervix and various mucosal mechanisms, including mucus, intact epithelium, and antimicrobial agents, protect the uterine environment from pathogens ascending the reproductive tract from the vagina. When these barriers are breached during parturition or insemination, bacterial pathogens can rapidly invade the uterus to establish infection, and this can result in clinical disease if cellular and humoral defense mechanisms are overwhelmed (Bondurant, 1999). Indeed, during and after parturition, bacteria readily ascend the female genital tract into the uterus. Although the majority of bacterial infection occurs in the postpartum animal, venereal transmitted pathogens, including *Tritrichomonas foetus* and *Campylobacter fetus,* can cause moderate persistent inflammation of the uterus and result in increased pregnancy losses (Corbeil et al., 1975; Schurig et al., 1975; Parsonson et al., 1976; Skirrow and BonDurant, 1990). Similarly, hygiene standards at AI have also been shown to impact bacterial contamination and to reduce the likelihood of pregnancy (Bas et al., 2011). It is estimated that about 40% of dairy cows develop clinical metritis following parturition, characterized by vaginal discharge of a brown foul-smelling watery discharge from the uterus, fever, reduced milk yield, and, in severe cases, toxemia (Markusfeld, 1987; Zwald et al., 2004). Most cases of metritis are resolved within 14 d of diagnosis with or without the use of antimicrobial therapy (Chenault et al., 2004). However, approximately 20% of the cows develop endometritis beyond 21 d postpartum, with a persistent purulent discharge from the uterus. Terminology defining endometritis is beginning to change as purulent vaginal mucus is not always correlated with the number of neutrophils detected in cytology samples collected from the surface of the endometrium by cytobrush (Dubuc et al., 2010). Furthermore, cytological endometritis has emerged as a problem of importance for dairy cattle reproduction because of reduced pregnancy per insemination and extended interval to pregnancy (Vieira-Neto et al., 2014). Animals suffering cytological endometritis present a persistent inflammatory uterine environment in the absence of clinical symptoms. The definitions of disease are set out in reviews (Sheldon et al., 2006, 2009). However, recent assessments of the literature indicate that some discrepancies remain between research teams as to the best practice for clinical diagnosis of both endometritis and metritis (Sannmann et al., 2012; de Boer et al., 2014). The financial impact of uterine disease in dairy cows has been estimated at approximately $650 million in the United States, with costs stemming from clinical treatment, lost milk production, culling for failure to conceive, and maintenance of replacement animals (Sheldon et al., 2009).

Although parturition is considered the event leading to uterine infection, several factors are associated with increasing the risk of uterine diseases. These include factors associated with uterine damage, such as dystocia, retained fetal membranes, twins, and stillbirth (Potter et al., 2010; Giuliodori et al., 2013). Parity has also been associated with uterine disease, with the greatest odds of developing metritis associated with either first parity or third and higher parities. It is surmised that primiparous animals are at increased risk of dystocia, whereas older third-parity animals are at high risk of retained fetal membranes, both resulting in uterine damage (Bruun et al., 2002). The dairy cow experiences a period of negative nutrient balance following parturition because of the high dietary demands for energy for milk synthesis. Energy homeostasis and metabolism are closely associated with the effectiveness of the immune system to combat infections (Mathis and Shoelson, 2011). Recent studies have given weight to the argument that cows with markers of more exacerbated tissue catabolism because of negative energy balance are more likely to develop uterine diseases (Silvestre et al., 2011; Giuliodori et al., 2013; Ribeiro et al., 2013).

In many instances, uterine infection and disease are common, and treatment is relatively straightforward. However, the consequences of infection and inflammation of the reproductive tract persist beyond the resolution of the clinical process, with marked depression in reproductive performance (Borsberry and Dobson, 1989; LeBlanc et al., 2002; Kasimanickam et al., 2004; Sheldon et al., 2009). Cows with clinical disease show a longer interval to estrus, irregular ovarian cycles, a prolonged postpartum luteal phase, delayed onset to ovarian cyclicity, and ultimately failure to conceive (Ribeiro et al., 2013).Compared to-normal animals, animals with endometritis experience a 1.7-fold increase in the culling rate (LeBlanc et al.,

2002). Treatment of cows with metritis uses routine administration of systemic antimicrobials, some of which have no milk discard requirements (Chenault et al., 2004), although the benefits of their use to improve reproductive performance remain to be demonstrated (Galvao et al., 2009). More recently, attempts have been made to produce vaccines to prevent metritis, and preliminary results are encouraging (Machado et al., 2014). However, even after clinical uterine disease has been resolved, these cows have a lower rate of pregnancy per AI than cows with no previous uterine disease (Borsberry and Dobson, 1989; LeBlanc et al., 2002; Kasimanickam et al., 2004; Sheldon et al., 2009). The mechanistic reasons behind continued infertility following resolution of infection (i.e., metritis) or resultant inflammation (i.e., endometritis) remain to be elucidated.

PATHOGENIC AGENTS INVOLVED IN UTERINE INFECTION

Microbial contamination of the uterus occurs shortly after parturition by a number of opportunistic bacteria, including *Escherichia coli, Trueperella pyogenes*, and the anaerobes *Prevotella* sp., *Fusobacterium necrophorum*, and *Fusobacterium nucleatum*. The first bacterial pathogen to colonize the upper reproductive tract following parturition involved in uterine disease is *E. coli* (Williams et al., 2007), and an endometrial-specific strain of *E. coli*, termed endometrial pathogenic *E. coli* (**EnPEC**), is distinct from gastrointestinal and extraintestinal pathogenic *E. coli* (Sheldon et al., 2010). Analysis of this specific strain revealed a surprising lack of virulence factors associated with EnPEC compared with pathogenic enteric and extraintestinal pathogenic *E. coli*. However, EnPEC possess an increased ability to adhere to and invade endometrial cells than other *E. coli* strains. Characterization of other metritis-associated bacteria, including some *E. coli* strains, has shown specific virulence factor expression such as the minor component of type 1 fimbriae, FimH (Sheldon et al., 2010; Bicalho et al., 2012). Endometrial pathogenic *E. coli* induce an endometrial inflammatory response due to the presence of the cell wall component lipopolysaccharide (**LPS**; i.e., an endotoxin). Endometrial-specific *E. coli* have now been sequenced, but the mechanism by which these bacteria preferentially establish disease in dairy cows is unclear beyond LPS-initiated inflammation (Goldstone et al., 2014b). It is interesting to note that recently, the degree to which *E. coli* is associated with uterine disease has come into question. Pyrosequencing for the microbiome of the uterus revealed a surprising absence of *E. coli* at 35 d in milk (**DIM**), whereas other studies have indicated a limited association with the presence of *E. coli* at this time point with uterine disease or infertility (Bicalho et al., 2010; Machado et al., 2012). However, it is important to realize the distinction between the presence of uterine *E. coli* at 35 DIM and the importance of uterine *E. coli* shortly after parturition where an association with uterine disease and infertility exists (Dohmen et al., 2000; Mateus et al., 2002; Sheldon et al., 2002; Williams et al., 2007; Sheldon et al., 2010; Prunner et al., 2014; Wagener et al., 2014).

Infection with *T. pyogenes* (formally *Arcanobacterium pyogenes*) is associated with the most severe cases of uterine inflammation in dairy cattle at d 26 or 40 postpartum (Bonnett et al., 1991; Prunner et al., 2014). *Trueperella pyogenes* elicits an inflammatory response in endometrial explants, increasing the inflammatory mediators IL-1β, IL-6, IL-8 and $PGF_{2\alpha}$ (Miller et al., 2007; Amos et al., 2014). However, much of the virulence of *T. pyogenes* is associated with the organism's secretion of a cholesterol-dependent cytolysin, pyolysin (**PLO**), which causes osmotic death of host cells. Exposure of endometrial stromal cells to PLO potently elicits cytolysis, although endometrial epithelial cells appear resistant to PLO-mediated lysis, probably because of the lower cholesterol content of epithelial cells than stromal cells (Amos et al., 2014). The differential cellular susceptibility to PLO reflects the observations that uterine damage is required for infection to cause disease, particularly when the protective epithelium is disrupted following parturition. Endometritis-causing *T. pyogenes* have now been fully sequenced, are highly similar among cows with uterine disease, and can produce experimentally induced infection resulting in clinical signs of endometritis with a purulent discharge in the uterus and vagina (Amos et al., 2014; Goldstone et al., 2014a).

The above-mentioned microbes dominate the literature regarding uterine disease, but it is important to consider the presence of less studied bacterial species associated with disease. The anaerobes *Prevotella* sp., *F. necrophorum*, and *F. nucleatum* have all been associated with severe uterine disease in cattle and appear to aid the pathogenesis of both *E. coli* and *T. pyogenes* (Olson et al., 1984). For example, *F. necrophorum* produces a leukotoxin that inactivates and kills leukocytes required to clear an infection (Narayanan et al., 2002). *Prevotella melaninogenica* has been shown to produce substances that inhibit phagocytosis of bacteria and induce the production of factors by the host immune system to cause tissue destruction (Jones and Gemmell, 1982; McGregor et al., 1986). It is interesting to note that other bacterial strains are also present in the uterus and are not associated with uter-

ine disease, such as *Staphylococci* and *Streptococci* (Williams et al., 2005). Furthermore, a wide variety of microbes can be identified in the postpartum uterus by molecular techniques, although their roles remain unclear (Machado et al., 2012).

TOLL-LIKE RECEPTOR SIGNALING

Bacteria utilize specialized virulence factors to cause tissue damage and promote disease, which leads to a host response to these bacteria directed by the innate immune system, including antimicrobial peptides, complement, and the Toll-like receptor (**TLR**) family. It is known that antimicrobial peptides and complement play a critical role in the initiation of inflammation and clearance of microbes from the uterus of cows (Bondurant, 1999); however, this review will focus on the role of TLR. Since Hoffman and Beutler identified the importance of Toll in flies and TLR in mammals for initiating the immediate response to pathogens (Lemaitre et al., 1996; Poltorak et al., 1998), the field of innate immunity has lavished a great deal of attention on this family of receptors and the role they play in disease.

In the cow there are 10 members of the TLR family (i.e., TLR1 to TLR10), each with the capability to bind specific conserved microbial components, although TLR10 has yet to be assigned a ligand. Each TLR has a specific cellular location dependent on the ligand to which it binds; TLR3, 7, 8, and 9 are intracellular, whereas the remaining members are principally cell surface receptors. The cell surface receptors mainly bind bacterial lipids, often bacterial cell wall components, whereas intracellular receptors bind nucleic acids, indicating a highly evolved mechanism to detect the presence of microbial agents dependent on their type and pathogenesis (Beutler, 2004). Of relevance to uterine disease, bacterial LPS binds to TLR4 in conjunction with the coreceptors lymphocyte antigen 96 (**LY96**, also known as **MD-2**) and cluster of differentiation (**CD**) 14, whereas bacterial lipopeptides are bound by TLR2 in concert with TLR1 or TLR6 (Cronin et al., 2012; Turner et al., 2014). Upon binding the receptor-specific ligand, an intracellular signaling cascade results in the expression of inflammatory mediators including the cytokines tumor-necrosis factor (**TNF**) α, interferon (**IFN**) γ, IL-1β, IL-6, and chemokines IL-8 and C-X-C motif chemokine (**CXCL**) 5 required for leukocyte infiltration and clearance of infectious agents (Kawai and Akira, 2010). In dairy cows, all 10 TLR are present in nonpregnant endometrium, whereas expression is variable in the postpartum endometrium but still present (Davies et al., 2008; Herath et al., 2009b; Table 1). Recently, it has been described that specific SNP in TLR2, 4, 6, and 9 have minor associations with uterine disease of dairy cows (Pinedo et al., 2013). These SNP may provide insight into the variability in disease susceptibility among cows exposed to the same bacteria. However, although the mechanisms of infection and disease are being uncovered, the underlying question remains: how do uterine infections in dairy cows result in infertility after the resolution of infection or disease?

Table 1. Toll-like receptor (TLR) ligands and endometrial expression[1]

Toll-like receptor	Ligand	Uterine expression	Endometrial cell type
TLR1	Bacterial lipoproteins	NP, postpartum	Epi, stroma
TLR2	Peptidoglycan, lipoteichoic acid and lipoprotein (most diverse)	NP, postpartum (increased in infertile animals*)	Epi, stroma
TLR3	Double stranded RNA	NP, postpartum	Epi, stroma
TLR4	Lipopolysaccharide	NP, postpartum (increased in infertile animals*)	Epi, stroma
TLR5	Flagellin	NP, postpartum	Epi
TLR6	Lipoteichoic acid, lipoproteins	NP, postpartum	Epi, stroma
TLR7	Single-stranded RNA	NP, postpartum	Epi, stroma
TLR8	Single-stranded RNA	NP, postpartum	ND
TLR9	Unmethylated CpG DNA	NP, postpartum	Epi, stroma
TLR10	Unknown	NP, postpartum	Stroma

[1]Exogenous ligands for the various TLR, their described expression in either nonpregnant or postpartum uterus biopsy, and cellular expression in purified endometrial epithelium or stroma. Abbreviations: NP = nonpregnant; Epi = epithelium; ND = not detected; an asterisk (*) denotes an increased expression in infertile compared to fertile animals. Information is derived from Davies et al. (2008), Herath et al. (2009b), and Kawai and Akira (2010).

MECHANISMS OF INFERTILITY CAUSED BY UTERINE INFECTION

We hypothesize that there are 3 main factors linking postpartum uterine infection of dairy cows with infertility, even following clearance of infectious agents. These are 1) disruption of endocrine signaling and the hypothalamic-pituitary-gonadal axis, 2) negative effects on the ability of the endometrium to support embryonic development and implantation, and 3) ovarian dysregulation resulting in reduced oocyte quality (Fig. 1).

Impact of Uterine Infection on Endocrine Signaling

It is curious to consider the impacts of uterine infection and inflammation on neuroendocrine signaling due to the spatial distance between the site of infection and the hypothalamus and pituitary at the base of the brain. However, experimental uterine LPS exposure

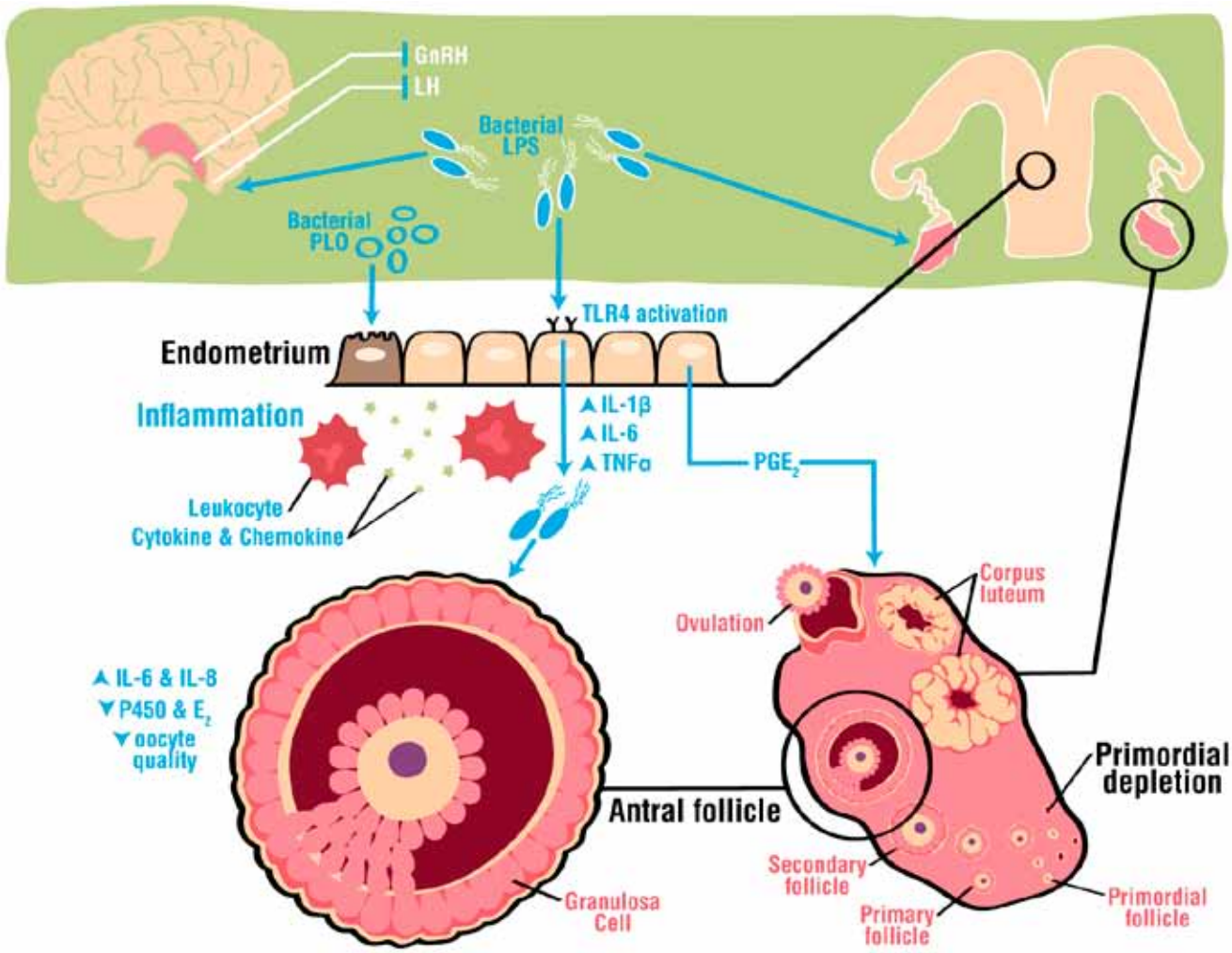

Figure 1. Schematic representation of uterine infection and impacts on the reproductive tract. This figure represents the all-encompassing effects of uterine bacterial infection on neuroendocrine signaling, uterine health, and ovarian function. Brain: GnRH and LH production are reduced. Endometrium: bacterial pyolysin (PLO) disrupts endometrial cells by osmotic lysis, whereas lipopolysaccharides (LPS) initiate an inflammatory response via Toll-like receptor (TLR) 4 activation, increasing cytokine, chemokine, and PGE_2 production. Ovary: the primordial follicle reserve is depleted, follicle growth is retarded, and luteal phase is prolonged. Ovarian granulosa cells respond to bacterial LPS in a TLR4-dependent manner, increasing inflammatory mediators, reducing aromatase and estradiol, and reducing oocyte competence. Illustration by Stacey Jones (University of Florida, Institute of Food and Agricultural Science).

in the postpartum cow or systemic administration in sheep decreases GnRH secretion by the hypothalamus and reduces LH pulsatility (Peter et al., 1989; Karsch et al., 2002). Furthermore, ovulation is delayed in cows following systemic or intramammary administration of LPS, although GnRH has been used therapeutically to induce normal ovarian cyclicity in these animals (Suzuki et al., 2001; Lavon et al., 2008). The specific mechanisms by which LPS exposure at a distant site affects hypothalamic-pituitary function have yet to be elucidated; experimental models of systemic LPS administration (described previously) indicate the possibility of LPS entering the circulation and traveling to the brain, although it is interesting that blockade of TLR4 signaling seems to prevent LPS-induced GnRH-LH signal disruption (Haziak et al., 2014).

The primary impact of disrupting endocrine signaling is the negative consequences on ovarian function. Cows with postpartum uterine infections within 2 wk after calving have reduced circulating estradiol and perturbed PG signaling, resulting in disruption of ovarian cyclicity, extended luteal phases, delayed ovulation, slower follicular growth, and increased risk of anovulation (Opsomer et al., 2000; Sheldon et al., 2002; Herath et al., 2007). The changes in follicular estradiol production are a direct result of reduced aromatase activity in granulosal cells due to LPS exposure (Price et al., 2013; Magata et al., 2014). Furthermore, LPS induces a switch of endometrial PG production from luteolytic $PGF_{2\alpha}$ to immune modulatory PGE_2 that likely contributes to the extension of the luteal phase (Herath et al., 2009a).

Endometrial Response to Uterine Infection

Uterine responsiveness to invading microbial pathogens must be rapid and robust to prevent the establishment of uterine infection. Proinflammatory genes such as *IL 1A*, *IL 1B*, *IL6*, *TNF,* and prostaglandin E synthase (**PTGES**) have been shown to be upregulated in the endometrium of animals with persistent endometritis compared with healthy cows (Herath et al., 2009b; Wathes et al., 2009; Fischer et al., 2010). These proinflammatory agents increase recruitment of neutrophils and macrophages to combat infection and aid in resolution of the disease (Sheldon et al., 2010). The S100 proteins have recently been described to contribute to the inflammatory function of neutrophils, macrophages, and mast cells (Goyette and Geczy, 2011). Endometrial expression of S100A8, S100A9, and S100A12 rapidly increases in response to the inflammatory mediators IL-6 and IL-10 induced by infection (Swangchan-Uthai et al., 2012). In addition, there is increased liver production of serum amyloid A and haptoglobin in cows with uterine disease after parturition (Sheldon et al., 2001). In vitro studies using endometrial explants or purified endometrial epithelial and stromal cells have shown similar inflammatory responses to *E. coli*, *T. pyogenes*, and highly purified bacterial cell wall components, LPS, lipoprotein, and peptidoglycan (Borges et al., 2012; Amos et al., 2014; Turner et al., 2014). Induction of proinflammatory mediators IL-1β, IL-6, and IL-8 has been shown to occur in these tissues in a TLR-dependent manner depending on the bacterial components utilized (Herath et al., 2006; Cronin et al., 2012; Turner et al., 2014). In combination, increased inflammatory mediators, cellular influx of immune cells, and induction of antimicrobial factors all work in concert to combat and clear the active uterine infection. However, as we have alluded to, this response may contribute to the infertility witnessed in cows following the resolution of infection. Indeed, when assessing endometrial expression of the inflammatory mediators *IL 1A* and *IL 1B* in cows with infection that became infertile, both mediators are expressed at greater abundance in infertile cows compared with those that remained fertile (Herath et al., 2009b). It is conceivable that an unchecked, excessive endometrial inflammatory response could contribute to infertility in cows following infection. One obvious mechanism is disruption of the preimplantation developmental environment. Many embryotrophic factors produced by the oviduct and endometrium are also immune modulators, and as such, excessive or inappropriate temporal expression of these factors may perturb embryonic development and negatively impact fertility. Indeed, when embryos are cultured in the presence of endometrial fluid from an inflamed uterus, total blastomere number and allocation to either inner cell mass or trophectoderm are negatively impacted (Hill and Gilbert, 2008). Similarly, one could surmise that embryo attachment and/or placentation could be equally affected by an inappropriately inflamed uterine environment.

Ovarian Response to Uterine Infection

When considering the mechanisms of infertility following uterine infection, the ovary is a logical target because it contains a vulnerable and finite reserve of oocytes required for subsequent generations, but how does infection at a distant site impact ovarian tissues? As described previously, ovarian function is perturbed following infection with reduced estradiol production, delayed ovulation, retarded follicular growth, and extended luteal phases. Beyond the direct effects of endocrine dysregulation on the ovary by altered LH patterns and shifts from endometrial $PGF_{2\alpha}$ to PGE_2 synthesis, cellular and molecular pathways within the ovarian follicle are also affected in cows suffering uterine infection. Key to the changes seen in the ovary is the presence of LPS within the follicular fluid of diseased cows (Herath et al., 2007). We are now beginning to understand the mechanisms by which the follicular environment, which is free of immune cells, can contribute to infertility in dairy cows.

Granulosal cells possess the molecular machinery required for detection of bacterial components, TLR, CD14, and MD-2. In addition, granulosal cells exposed to the bacterial components LPS or peptidoglycan mount an acute inflammatory response by increased production of inflammatory mediators IL-1β, IL-6, IL-8, and TNFα (Herath et al., 2007; Bromfield and Sheldon, 2011; Price et al., 2013; Price and Sheldon, 2013). The granulosal cell inflammatory response to LPS is initiated by TLR4, and intracellular signaling occurs through rapid phosphorylation of the extracellular-signal-regulated kinases (**ERK**) and p38 kinase pathways (Bromfield and Sheldon, 2011; Price et al., 2013). Estradiol production by granulosal cells is reduced following LPS exposure by reducing aromatase expression; however, the mechanism of aromatase reduction is unclear (Herath et al., 2007). Of paramount interest is the finding that oocyte maturation is also perturbed in the presence of LPS (Bromfield and Sheldon, 2011).

Oocyte maturation occurs spontaneously in vitro, developing from the germinal vesicle stage to the metaphase II (**MII**) stage in approximately 24 h. This highly orchestrated development matures both the nuclear and cytoplasmic compartments of the oocyte for the first cellular divisions of the early embryo. Pro-

duction of IL-6 by cumulus oocyte complexes (**COC**) is increased in response to LPS in vitro. Work by our group has shown that oocyte maturation in the presence of LPS at concentrations comparable to those found within the follicle significantly reduces the developmental competence of the oocyte, increasing germinal vesicle breakdown failure and causing abnormal spindle formation (Bromfield and Sheldon, 2011). Maturation of the COC required for ovulation is also perturbed, with LPS inducing cumulus expansion in the absence of gonadotropin signaling (Bromfield and Sheldon, 2011). In addition, maturation of bovine oocytes in the presence of LPS reduced blastocyst development rate, whereas embryos cultured in the presence of LPS have no adverse effects on blastocyst development (Soto et al., 2003). In the mouse it has been shown that TLR4 plays a physiological role in COC expansion by binding the endogenous ligand hyaluronan, inducing IL-6 expression and promoting matrix expansion (Shimada et al., 2006, 2008). However, the mechanism by which LPS reduces oocyte quality has yet to be understood. It is possible that LPS directly influences oocyte development, although it seems more plausible that LPS dysregulates inflammatory mediators required for oocyte development. The physiological importance of cytokines in oocyte development and ovarian function is well established (Espey, 1980; Richards et al., 2008; Spanel-Borowski, 2011). Immunological factors, such as IL-6, colony-stimulating factor 2, leukemia inhibitory factor, IGF-I, TNFα, growth differentiation factor 9, bone morphogenetic protein 15, and epidermal growth factor, are all critical to oocyte development, and their expression has the potential to be altered during infection (Spicer et al., 1988, 2006; Alpizar and Spicer, 1994; Dong et al., 1996; Spicer, 1998; Yan et al., 2001; Molyneaux et al., 2003; Van Slyke et al., 2005; Hansen et al., 2014). Redundancies in the mediators between oocyte development and inflammation, as well as alterations in their abundance, are more likely mechanisms by which oocyte development is perturbed because of bacterial infection. The intracellular signaling pathways utilized by these various signaling moieties use the central phosphatidylinositol-4,5-bisphosphate 3-kinase (**PI3K**)–protein kinase B (**AKT**) pathway critical for oocyte maturation (Okumura et al., 2002; Van Slyke et al., 2005). It is currently unclear whether the presence of bacterial pathogens alters these intracellular pathways in oocytes, reducing developmental competence of oocytes by disrupting cytoplasmic or nuclear maturation.

The negative effects of LPS on developing oocytes in the dominant follicle explain infertility shortly after infection, but how is long-term infertility in animals following uterine infection explained? We propose that infection also perturbs smaller developing follicles, including primordial stage follicles. Initiation of folliculogenesis is a tightly orchestrated series of molecular and cellular events. Primordial follicles containing an immature oocyte at the dictyotene stage of meiosis are held in a quiescent state by the presence of inhibitor factors, including phosphatase and tensin homolog (**PTEN**) and forkhead box O3a (**FOXO3a**; Castrillon et al., 2003; Reddy et al., 2008; Bao et al., 2011). In vitro culture of cortical ovarian explant results in the unexplained spontaneous activation of the primordial follicle pool to develop primary and secondary follicles. In the presence of LPS, primordial follicle activation is increased, resulting in an enlarged pool of primary follicles and a depletion of the primordial follicle reserve (Bromfield and Sheldon, 2013). In vivo studies using mice revealed a similar decrease in the primordial follicle reserve in conjunction with an increase in follicle atresia after administration of LPS. The LPS-induced activation of the primordial pool occurs in conjunction with loss of PTEN and FOXO3a protein in primordial follicles, but it is unclear if this is causative or resultant of follicle activation. Ovarian explant cultures increase production of inflammatory mediators IL-1β, IL-6, and IL-8 in response to LPS. It was interesting to note the high basal level of IL-6 production in ovarian explants cultured in control medium, which we propose is involved in the spontaneous activation of primordial follicles. Assessment of larger preantral follicle responses to LPS reveals that these stages, at least in vitro, appear to be resistant to the effects of LPS, with no change in estradiol production or oocyte and follicle growth (Bromfield and Sheldon, 2013). Although the studies described previously investigate the role of LPS on follicle growth in vitro, they indicate that fertility may be affected in both short- and long-term scenarios with the primordial follicle pool inappropriately activated impacting long-term fertility, whereas low-quality oocytes from dominate follicles may impact short-term fertility. The precise mechanisms by which LPS exposure reduces the primordial follicle pool and oocyte development remain to be elucidated, but we propose that alterations in redundant signaling pathways integral in both immunity and development could play a major role. As in oocyte development, the PI3K-AKT pathway is critical for coordinated recruitment of primordial follicles (Wandji et al., 1996; Fortune, 2003). Similarly, activation of the TLR signaling also uses the central PI3K-AKT pathway to increase production of inflammatory mediators such as IL-6, feeding back into the pathway for increased stimulation (Laird et al., 2009). We propose that activation of the TLR4 and IL-6 path-

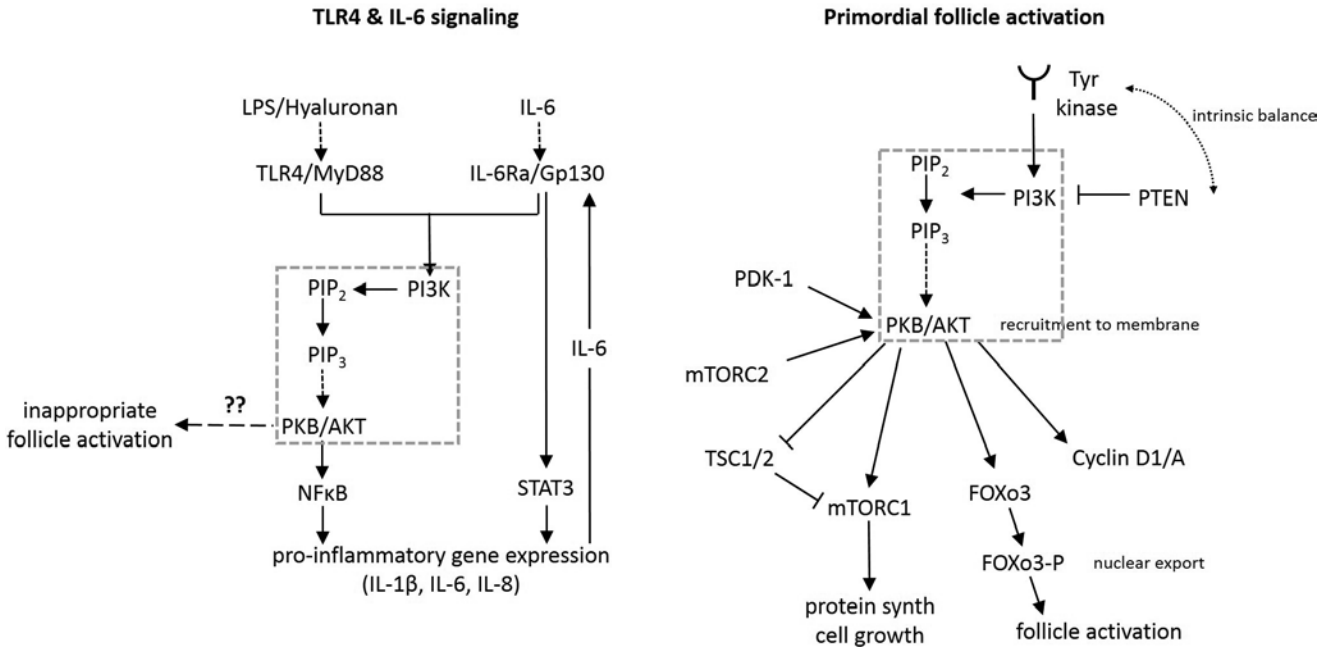

Figure 2. Schematic representation of the redundancies between Toll-like receptor (TLR) 4/IL-6 signaling and the cellular pathways in granulosa cells and oocytes involved in primordial follicle activation. The left panel shows the intracellular pathways of TLR4 or IL-6 activation through phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and protein kinase B (AKT). The right panel shows the process of primordial follicle activation utilizing the same PI3K and AKT pathway, which is regulated by the balance of phosphatase and tensin homolog (PTEN) activation by tyrosine kinase receptors. We propose that bacterial activation of the TLR/IL-6 pathway (left) contributes to inappropriate activation of the follicle activation pathway (right). LPS, Lipopolysaccharides; MyD88, Myeloid differentiation primary response gene 88; IL-6Ra, Interleukin 6 receptor alpha; Gp130, Glycoprotein 130; STAT3, Signal transducer and activator of transcription 3; NFκβ, nuclear factor kappa-light-chain-enhancer of activated B cells; PIP, phosphatidylinositol 3,4,5 trisphosphate; IL, Interleukin; PDK-1, Phosphoinositide-dependent kinase-1; mTORC1/2, mammalian target of rapamycin complex 1 & 2; TSC1/2, tuberous sclerosis 1; FOXO3, Forkhead box O3.

ways by bacterial pathogens contributes to the inappropriate recruitment of primordial follicles by activation of the same pathway (Fig. 2). Exploring the links between uterine disease and the ovary will require exploiting animal models.

ANIMAL MODELS OF UTERINE INFECTION AND THEIR IMPLICATIONS

In 1929, Nobel laureate August Krogh coined the principle that "for such a large number of problems there will be some animal of choice on which it can be most conveniently studied" (Krogh, 1929; Albertini, 2011). Krogh's principle encompasses the development of PCR using thermostable *Taq* polymerase from heat-labile bacteria (Chien et al., 1976), the study of menstruation in the short-tailed fruit bat (Rasweiler and de Bonilla, 1992), and the utilization of jellyfish green fluorescent protein in cell biology (Chalfie et al., 1994). Unique to animal science, we have ready access to the animals in which the problem is relevant. However, uterine infection in dairy cows shares a number of similarities with puerperal fever and pelvic inflammatory disease (**PID**) in women. Women who suffer PID do so as a result of uterine infection usually bought on by sexually transmitted bacterial infections such as *Gonorrhea* and *Chlamydia* (Ross, 2002). Pelvic inflammatory disease causes pain and infertility and is the leading cause of gynecological hospitalization of women in the developed world (Ross, 2002). Studies in women indicate that PID causes ovarian changes similar to those seen in the dairy cow following uterine infection (Weiner and Wallach, 1974; Margolis, 1976; Bychkov, 1990). In addition, studies of infection and immunity using primary cells from the bovine uterus and ovary are similar to studies using human endometrial and ovarian cells (Sanchotello et al., 1992; Allhorn et al., 2008; Price et al., 2012). The similarities between human PID and bovine uterine disease give us a unique opportunity to understand a disease state pertinent to both agricultural production and human health. For humans, the impact of uterine disease might be particularly important for patients who have unexplained infertility and/or a need for assisted reproduction techniques, such as in vitro fertilization, where the immune environment requires a balance between physiological (for normally ovarian function) and pathological (to combat bacterial infection) responses (Chegini et al., 2002; Li et al., 2006). The availability of bovine oocytes and granulosa cells may provide an opportunity to inform human studies relevant to puerperal fever and PID in women.

Several animal models of uterine infection have been developed with varying degrees of success in reproducing the disease. The mouse has commonly been used by infusing bacteria into the uterus. We have used the mouse as a convenient model by administering LPS intraperitoneally and noted that although primordial depletion occurs, it is likely due to follicle atresia as opposed to increased primordial follicle activation, as in the cow (Bromfield and Sheldon, 2013). In the dairy cow it has long been established that the endocrine status of the animal is key to the development and severity of uterine infection following infusion of *E. coli* and *T. pyogenes*. Induction of the disease at estrus or administration of exogenous estradiol limits the formation of infection, whereas bacterial infusion during the luteal phase or exogenous progesterone administration increases the likelihood of uterine infection persisting (Rowson et al., 1953; Ayliffe and Noakes, 1982). In addition, the structural integrity of the endometrium is important; as noted previously, physical damage to the epithelial layer seems to be important in establishing disease. Uterine infusion of the *T. pyogenes* virulence factor PLO without endometrial damage results in no signs of disease (Miller et al., 2007). However, uterine infusion of *T. pyogenes* with mechanical disruption of the endometrium results in uterine disease (Amos et al., 2014). The potential to exploit the dairy cow model of uterine disease has yet to be fully appreciated in regard to human disease, particularly for study of the impacts on the ovary.

SUMMARY AND CONCLUSIONS

Uterine infection and inflammation in the dairy cow cause infertility. However, we are only now beginning to understand the importance of pathological ovarian dysfunction that persists beyond the duration of infection. The effects of infection on uterine and neuroendocrine homeostasis are well established; however, the extended temporal development of the follicle and oocyte lends the ovary to prolonged vulnerability following infectious challenge, resulting in perturbations that may not manifest until sometime after disease resolution. Primordial follicle quiescence to ovulation all appear susceptible to perturbation by infections of the reproductive tract, yet little is known about the mechanisms responsible for causing infertility in dairy cows. Further work using a bovine model that recapitulates the bacterial infection and persistent inflammation of uterine disease is urgently needed to elucidate the pathways and mechanisms responsible for ovarian dysfunction following uterine infection. Appropriate animal models will allow the development of strategies to limit the impact of disease on the dairy industry and will provide valuable insight into human health and fertility.

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