RUNNING HEAD: Uterine infection and infertility in dairy cows

Uterine infection: Linking infection and innate immunity with infertility in the high producing dairy cow[1]

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ABSTRACT: Uterine contamination with bacteria is ubiquitous in the postpartum dairy cow. Nearly half of all postpartum dairy cows develop clinical disease resulting in metritis and endometritis, which causes 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 finally 3) impacts of infection on ovarian function and oocyte/ follicle development. Normal ovarian follicle 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

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, which gives results in the failure of the anatomical barriers to prevent ascending uterine bacterial infiltration. While bacterial infections are normally cleared within 3 to 5 wk of parturition, many cows display signs of impaired fertility, including reduced conception, lower submission rates, and increased calving to conception intervals, and these signs are 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 clearing 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.

POST-PARTUM 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. While 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

60 the uterus and result in increased pregnancy losses (Corbeil et al., 1975; Schurig et al., 1975;
61 Parsonson et al., 1976; Skirrow and BonDurant, 1990). Similarly, hygiene standards at AI have
62 also been shown impact bacterial contamination and to reduce the likelihood of pregnancy (Bas
63 et al., 2011). It is estimated that about 40% of dairy cows develop clinical metritis following
64 parturition, characterized by vaginal discharge of a brown foul-smelling watery discharge from
65 the uterus, fever, reduced milk yield and, in severe cases, toxemia (Markusfeld, 1987; Zwald et
66 al., 2004). Most cases of metritis are resolved within 14 d of diagnosis with or without the use of
67 antimicrobial therapy (Chenault et al., 2004). However, approximately 20% of the cows develop
68 endometritis beyond 21 d postpartum, with a persistent purulent discharge from the uterus.
69 Terminology defining endometritis is beginning to change as purulent vaginal mucus is not
70 always correlated with the number of neutrophils detected in cytology samples collected from
71 the surface of the endometrium by cytobrush (Dubuc et al., 2010). Furthermore, cytological
72 endometritis has emerged as a problem of importance for dairy cattle reproduction because of
73 reduced pregnancy per insemination and extended interval to pregnancy (Vieira-Neto et al.,
74 2014). Animals suffering cytological endometritis present a persistent inflammatory uterine
75 environment in the absence of clinical symptoms. The definitions of disease are set out in
76 reviews (Sheldon et al., 2006; Sheldon et al., 2009). However, recent assessments of the
77 literature suggest some discrepancies remain between research teams as to the best practice for
78 clinical diagnosis of both endometritis and metritis (Sannmann et al., 2012; de Boer et al., 2014).
79 The financial impact of uterine disease in dairy cows has been estimated at approximately $650
80 million in the U.S., with costs stemming from clinical treatment, lost milk production, culling for
81 failure to conceive and maintenance of replacement animals (Sheldon et al., 2009).
82 While parturition is considered the event leading to uterine infection, several risk 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 highest odds of developing metritis associated with either first, or greater than or equal to third parity. It is surmised that primiparous animals are at increased risk of dystocia while 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 is 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 with normal cows, endometritis results in a 1.7-fold increase in the culling rate of animals (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, after the resolution of clinical disease, cows still have reduced pregnancy/ AI (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 (metritis), or resultant inflammation (endometritis), remains 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 endometrial specific strain of *E. coli*, termed endometrial pathogenic *E. coli* (**EnPEC**) are distinct from gastro-intestinal and extra-intestinal 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 extra-intestinal pathogenic *E. coli*. However, EnPEC possess an increased ability to adhere and invade endometrial cells than other *E. coli* strains. Characterization of other metritis associated bacteria, including some *E. coli* strains, have shown specific virulence factor expression like 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

129 cows is unclear beyond LPS initiated inflammation (Goldstone et al., 2014b). It is interesting to
130 note that recently the degree to which *E. coli* is associated with uterine disease has come into
131 question. Pyrosequencing for the microbiome of the uterus revealed a surprising absence of *E.*
132 *coli* at 35 DIM, while other studies have suggested a limited association with the presence of *E.*
133 *coli* at this time point with uterine disease or infertility (Bicalho et al., 2010; Machado et al.,
134 2012). However, it is important to realize the distinction between the presence of uterine *E. coli*
135 at 35 days in milk (**DIM**) and the importance of uterine *E. coli* shortly after parturition where an
136 association with uterine disease and infertility exists (Dohmen et al., 2000; Mateus et al., 2002;
137 Sheldon et al., 2002; Williams et al., 2007; Sheldon et al., 2010; Prunner et al., 2014; Wagener et
138 al., 2014).

139 Infection with *T. pyogenes* (formally *Arcanobacterium pyogenes*) is associated with the
140 most severe cases of uterine inflammation in dairy cattle at day 26 or 40 postpartum (Bonnett et
141 al., 1991; Prunner et al., 2014). *Trueperella pyogenes* elicits an inflammatory response in
142 endometrial explants, increasing the inflammatory mediators IL-1β, IL-6, IL-8 and $PGF_{2\alpha}$ (Miller
143 et al., 2007; Amos et al., 2014). However, much of the virulence of *T. pyogenes* is associated
144 with the organism's secretion of a cholesterol dependent cytolysin, pyolysin (**PLO**), which
145 causes osmotic death of host cells. Exposure of endometrial stromal cells to PLO potently elicits
146 cytolysis, although endometrial epithelial cells appear resistant to PLO-mediated lysis, probably
147 due to the lower cholesterol content of epithelial than stromal cells (Amos et al., 2014). The
148 differential cellular susceptibility to PLO reflects the observations that uterine damage is
149 required for infection to cause disease, particularly when the protective epithelium is disrupted
150 following parturition. Endometritis-causing *T. pyogenes* have now been fully sequenced and are
151 highly similar amongst 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 lesser 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 which inactivates and kills leukocytes required to clear an infection (Narayanan et al., 2002). *Prevotella melaninogenica* has been shown to produces substances which 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 uterine 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 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;

175 Poltorak et al., 1998), the field of innate immunity has lavished a great deal of attention on this
176 family of receptors and the role they play in disease.

177 In the cow there are 10 members of the TLR family (TLR1 to 10), each with the
178 capability to bind specific conserved microbial components, although TLR10 has yet to be
179 assigned a ligand. Each TLR has a specific cellular location dependent on the ligand to which it
180 binds; TLR 3, 7, 8 and 9 are intracellular, whereas the remainder are principally cell surface
181 receptors. The cell surface receptors mainly bind bacterial lipids, often bacterial cell wall
182 components, whereas intracellular receptors bind nucleic acids, indicating a highly evolved
183 mechanism to detect the presence of microbial agents dependent on their type and pathogenesis
184 (Beutler, 2004). Of relevance to uterine disease, bacterial LPS binds to TLR4 in conjunction with
185 the co-receptors lymphocyte antigen 96 (**LY96**, also known as **MD-2**) and cluster of
186 differentiation (**CD**) 14, whilst bacterial lipopeptides are bound by TLR2 in concert with TLR1
187 or TLR6 (Cronin et al., 2012; Turner et al., 2014). Upon binding the receptor specific ligand, an
188 intracellular signaling cascade results in the expression of inflammatory mediators including the
189 cytokines tumor-necrosis factor (**TNF**)-α, interferon (**IFN)-**γ, IL-1β, IL-6, and chemokines IL-8
190 and C-X-C motif chemokine (**CXCL**) 5 required for leukocyte infiltration and clearance of
191 infectious agents (Kawai and Akira, 2010). In dairy cows, all 10 TLR are present in non-
192 pregnant endometrium, whereas expression is variable in the postpartum endometrium but still
193 present (Davies et al., 2008; Herath et al., 2009b) (**Table 1**). Recently it has been described that
194 specific SNP in TLR 2, 4, 6 and 9 have minor associations with uterine disease of dairy cows
195 (Pinedo et al., 2013). These SNP may provide an insight into the variability in disease
196 susceptibility between cows exposed to the same bacteria. However, whilst the mechanisms of
197 infection and disease are being uncovered, the underlying question remains; how do uterine

198 infections in dairy cows result in infertility after the resolution of infection or disease?

199 ## MECHANISMS OF INFERTILITY CAUSED BY UTERINE INFECTION

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

205 ### *Impact of Uterine Infection on Endocrine Signaling*

206 It is curious to consider the impacts of uterine infection and inflammation on neuro-
207 endocrine signaling due to the spatial distance between the site of infection and the
208 hypothalamus and pituitary on the base of the brain. However, experimental uterine LPS
209 exposure in the postpartum cow or systemic administration in the sheep decreases GnRH
210 secretion by the hypothalamus and reduces LH pulsatility (Peter et al., 1989; Karsch et al., 2002).
211 Furthermore, ovulation is delayed in cows following systemic or intramammary administration
212 of LPS, although GnRH has been used therapeutically to induce normal ovarian cyclicity in these
213 animals (Suzuki et al., 2001; Lavon et al., 2008). The specific mechanisms by which LPS
214 exposure at a distant site impacts hypothalamus-pituitary function has yet to be elucidated,
215 experimental models of systemic LPS administration (above) suggest the possibility of LPS
216 entering the circulation and traveling to the brain, while it is interesting that blockade of TLR4
217 signaling seems to prevent LPS-induced GnRH-LH signal disruption (Haziak et al., 2014).

218 The primary impact of disrupting endocrine signaling are the negative consequences on
219 ovarian function. Cows with postpartum uterine infections within two weeks of calving have

reduced circulating estradiol and perturbed prostaglandin signaling resulting in disruption of ovarian cyclicity, extended luteal phases, delayed ovulation, slower follicle 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 granulosa 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. Pro-inflammatory genes such as *IL-1α*, *IL-1β*, *IL-6*, *TNFα* and *PTGES* have been shown to be upregulated in the endometrium of animals with persistent endometritis compared to healthy cows (Herath et al., 2009b; Wathes et al., 2009; Fischer et al., 2010). These pro-inflammatory agents increase recruitment of neutrophils and macrophages to combat infection and aid in resolution (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 have all 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-1α* and *IL-1β* in cows with infection which became infertile, both mediators are expressed at higher abundance in infertile cows compared with those which 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 mechanisms is disruption of the pre-implantation 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 blasomere number and allocation are negatively impacted (Hill and Gilbert, 2008). Similarly one could surmise that embryo attachment and\or placentation could be equally effected 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 as 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 above, ovarian function is perturbed following infection with reduced estradiol production, delayed

ovulation, retarded follicle 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.

Granulosa cells possess the molecular machinery required for detection of bacterial components, TLR, CD14 and MD-2. In addition, granulosa 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 granulosa 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 granulosa 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. Production of IL-6 by cumulus oocyte complexes (**COC**)

289 is increased in response to LPS *in vitro*. Work by our group has shown that oocyte maturation in
290 the presence of LPS at concentrations comparable to those found within the follicle significantly
291 reduces the developmental competence of the oocyte, increasing germinal vesicle breakdown
292 failure, and causing abnormal spindle formation (Bromfield and Sheldon, 2011). Maturation of
293 the COC required for ovulation is also perturbed, with LPS inducing cumulus expansion in the
294 absence of gonadotropin signaling (Bromfield and Sheldon, 2011). In addition, maturation of
295 bovine oocytes in the presence of LPS reduced blastocyst development rate, while embryos
296 cultured in the presence of LPS have no adverse effects on blastocyst development (Soto et al.,
297 2003). In the mouse it has been shown that TLR4 plays a physiological role in COC expansion
298 by binding the endogenous ligand hylauronan, inducing IL-6 expression and matrix expansion
299 (Shimada et al., 2006; Shimada et al., 2008). However, the mechanism by which LPS reduces
300 oocyte quality is yet to be understood. It is possible that LPS directly influence oocyte
301 development, although it seems more plausible that LPS dysregulates inflammatory mediators
302 required for oocyte development. The physiological importance of cytokines in oocyte
303 development and ovarian function is well established (Espey, 1980; Richards et al., 2008;
304 Spanel-Borowski, 2011). Immunological factors such as IL-6, colony-stimulating factor 2,
305 leukemia inhibitory factor, IGF-I, TNFα, growth differentiation factor 9, bone morphogenetic
306 protein 15, and epidermal growth factor are all critical to oocyte development and their
307 expression has the potential to be altered during infection (Spicer et al., 1988; Alpizar and
308 Spicer, 1994; Dong et al., 1996; Spicer, 1998; Yan et al., 2001; Molyneaux et al., 2003; Van
309 Slyke et al., 2005; Spicer et al., 2006; Hansen et al., 2014). Redundancies in the mediators
310 between oocyte development and inflammation and alterations in their abundance are more
311 likely mechanisms by which oocyte development is perturbed due to bacterial infection. The

intracellular signaling pathways utilized by these various signaling moieties uses 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 explains infertility shortly after infection, but how is long-term infertility explained in animals following uterine infection? 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 pre-antral follicle responses to LPS reveal 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 above studies investigate the role of LPS on follicle growth *in vitro*, they suggest that fertility may be affected in both short and long term scenarios with the primordial follicle pool inappropriately activated impacting long-term fertility, while 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 like IL-6, feeding back into the pathway for increased stimulation (Laird et al., 2009). We propose that activation of the TLR4/IL-6 by bacterial pathogens contributes to the inappropriate recruitment of primordial follicles by activation of the same pathway (**Figure 2**). To explore 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 thermo stable Tap 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 to 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 suggest 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) response (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 been commonly used by infusing bacteria into the uterus. We have used the mouse as a convenient model by administering LPS intraperitoneally and noted that while 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 above 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 causes infertility. However, we are only now beginning to understand the importance of pathological ovarian dysfunction which persists beyond the duration of infection. The effects of infection on uterine and neuro-endocrine 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 which 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

404 inflammation of uterine disease is urgently needed to elucidate the pathways and mechanisms
405 responsible for ovarian dysfunction following uterine infection. Appropriate animal models will
406 allow the development of strategies to limit the impact of disease on the dairy industry and
407 provide valuable insight into human health and fertility.

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739 **Table 1**. Toll-like receptor ligands and endometrial expression.

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 (↑ in infertile animals*)	Epi, stroma
TLR3	Double stranded RNA	NP, postpartum	Epi, stroma
TLR4	Lipopolysaccharide	NP, postpartum (↑ 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

740 Exogenous ligands for the various TLR, their described expression in either non-pregnant or
741 postpartum uterus biopsy, and cellular expression in purified endometrial epithelium or stroma.
742 Abbreviations: NP = non-pregnant; Epi = epithelium; ND = not detected; * denotes an increased
743 expression in infertile compared to fertile animals. Information is derived from (Davies et al.,
744 2008; Herath et al., 2009b; Kawai and Akira, 2010).

FIGURE CAPTIONS

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, while lipopolysaccharides (**LPS**) initiates 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 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, UF/IFAS.

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).

765

Figure 1

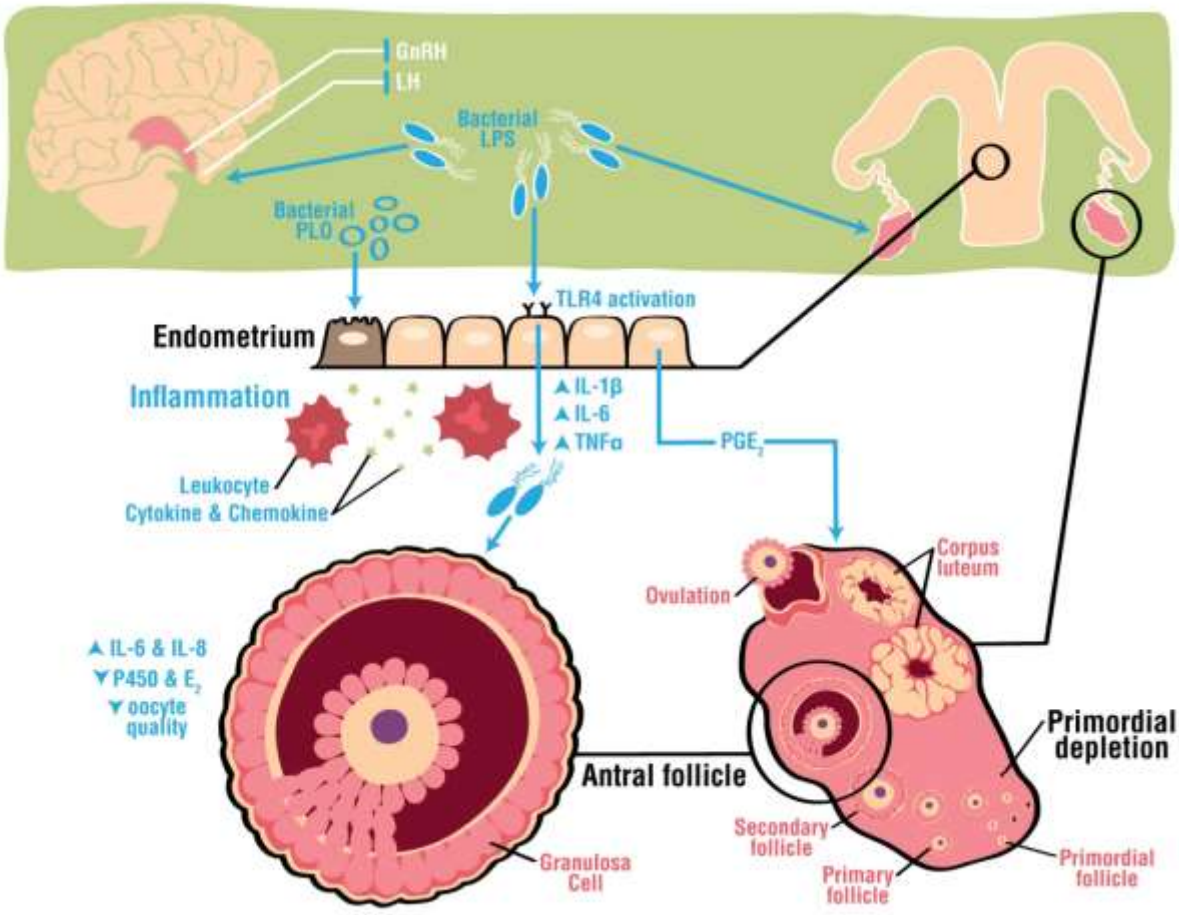
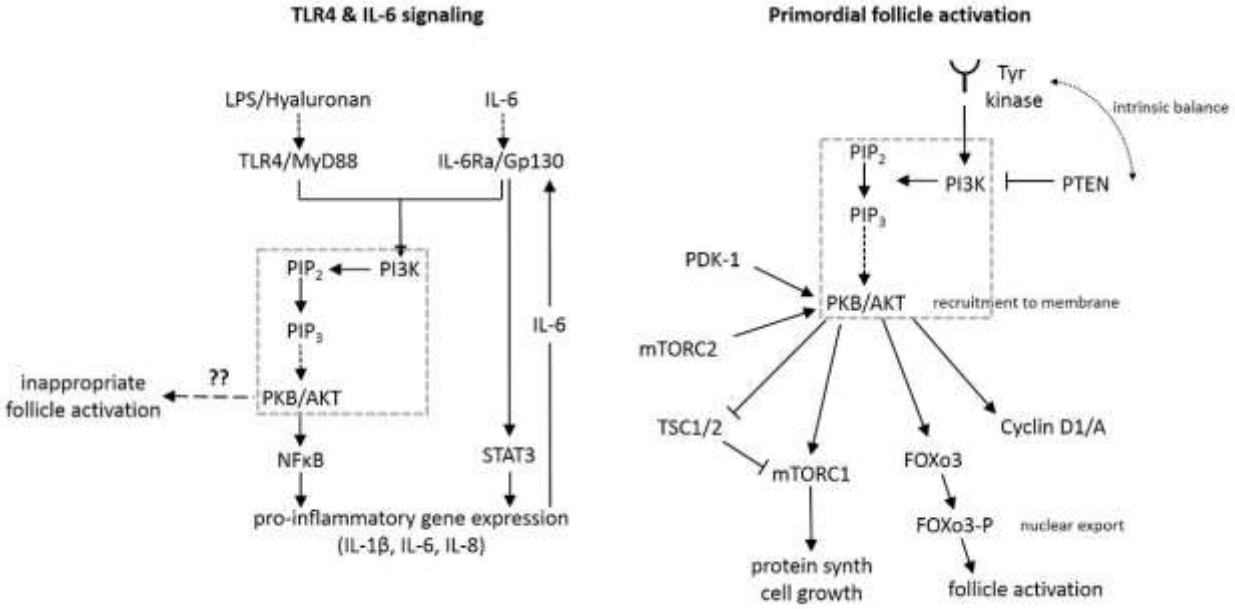

GnRH
LH
Bacterial LPS
Bacterial PLO
TLR4 activation
Endometrium
Inflammation
▲IL-1β
▲IL-6
▲TNFα
PGE2
Leukocyte
Cytokine & Chemokine
Corpus luteum
Ovulation
▲IL-6 & IL-8
▼P450 & E2
▼oocyte quality
Primordial depletion
Antral follicle
Secondary follicle
Primordial follicle
Primary follicle
Granulosa Cell

Figure 2


TLR4 & IL-6 signaling
LPS/Hyaluronan
IL-6
TLR4/MyD88
IL-6Ra/Gp130
PIP2
PI3K
PIP3
??
inappropriate
follicle activation
PKB/AKT
NFκB
STAT3
IL-6
pro-inflammatory gene expression
(IL-1β, IL-6, IL-8)
Primordial follicle activation
Tyr
kinase
intrinsic balance
PIP2
PI3K
PTEN
PIP3
PDK-1
PKB/AKT
recruitment to membrane
mTORC2
TSC1/2
Cyclin D1/A
mTORC1
FOXo3
FOXo3-P
nuclear export
protein synth
cell growth
follicle activation