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1 **RUNNING HEAD:** Uterine infection and infertility in dairy cows

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5 **Uterine infection: Linking infection and innate immunity with infertility in**
6 **the high producing dairy cow¹**

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

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

32

INTRODUCTION

33 The uterine mucosal environment is protected from pathogenic bacterial infiltration by
34 physical anatomical barriers and active molecular mechanisms. However, in comparison to non-
35 dairy cattle breeds, high-milk-yield dairy breeds such as the Holstein-Friesian are prone to
36 uterine bacterial contamination when these mechanisms fail, primarily following parturition. In

37 the dairy cow, parturition generates considerable tissue damage to the endometrium and cervix,
38 which gives results in the failure of the anatomical barriers to prevent ascending uterine bacterial
39 infiltration. While bacterial infections are normally cleared within 3 to 5 wk of parturition, many
40 cows display signs of impaired fertility, including reduced conception, lower submission rates,
41 and increased calving to conception intervals, and these signs are temporally after the resolution
42 of the signs of uterine disease (Borsberry and Dobson, 1989; McDougall, 2001; LeBlanc et al.,
43 2002). The innate immune response to bacteria is key to rapidly clearing infection (Herath et al.,
44 2006; Davies et al., 2008; Cronin et al., 2012; Turner et al., 2014). Recruitment of hematopoietic
45 immune cells, and the inflammatory response including secretion of cytokine and chemokines,
46 all combine to clear the bacterial infection and restore hemostatic function of the endometrium
47 (Sheldon and Roberts, 2010). However, evidence is emerging that these inflammatory events
48 have long-term consequences on the fertility of dairy cows by negatively impacting endocrine
49 signaling, uterine homeostasis, and ovarian function.

50 **POST-PARTUM DISEASE IN THE DAIRY COW**

51 Under normal circumstances, the anatomical barrier of the cervix and various mucosal
52 mechanisms including mucus, intact epithelium, and antimicrobial agents protect the uterine
53 environment from pathogens ascending the reproductive tract from the vagina. When these
54 barriers are breached during parturition or insemination, bacterial pathogens can rapidly invade
55 the uterus to establish infection, and this can result in clinical disease if cellular and humoral
56 defense mechanisms are overwhelmed (Bondurant, 1999). Indeed, during and after parturition,
57 bacteria readily ascend the female genital tract into the uterus. While the majority of bacterial
58 infection occurs in the postpartum animal, venereal transmitted pathogens including
59 *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

83 are associated with increasing the risk of uterine diseases. These include factors associated with
84 uterine damage, such as dystocia, retained fetal membranes, twins, and stillbirth (Potter et al.,
85 2010; Giuliodori et al., 2013). Parity has also been associated with uterine disease, with the
86 highest odds of developing metritis associated with either first, or greater than or equal to third
87 parity. It is surmised that primiparous animals are at increased risk of dystocia while older third-
88 parity animals are at high risk of retained fetal membranes, both resulting in uterine damage
89 (Bruun et al., 2002). The dairy cow experiences a period of negative nutrient balance following
90 parturition because of the high dietary demands for energy for milk synthesis. Energy
91 homeostasis and metabolism are closely associated with the effectiveness of the immune system
92 to combat infections (Mathis and Shoelson, 2011). Recent studies have given weight to the
93 argument that cows with markers of more exacerbated tissue catabolism because of negative
94 energy balance are more likely to develop uterine diseases (Silvestre et al., 2011; Giuliodori et
95 al., 2013; Ribeiro et al., 2013).

96 In many instances, uterine infection and disease is common and treatment is relatively
97 straightforward. However, the consequences of infection and inflammation of the reproductive
98 tract persist beyond the resolution of the clinical process, with marked depression in reproductive
99 performance (Borsberry and Dobson, 1989; LeBlanc et al., 2002; Kasimanickam et al., 2004;
100 Sheldon et al., 2009). Cows with clinical disease show a longer interval to estrus, irregular
101 ovarian cycles, a prolonged postpartum luteal phase, delayed onset to ovarian cyclicity, and
102 ultimately failure to conceive (Ribeiro et al., 2013). Compared with normal cows, endometritis
103 results in a 1.7-fold increase in the culling rate of animals (LeBlanc et al., 2002). Treatment of
104 cows with metritis uses routine administration of systemic antimicrobials, some of which have
105 no milk discard requirements (Chenault et al., 2004), although the benefits of their use to

106 improve reproductive performance remain to be demonstrated (Galvao et al., 2009). More
107 recently, attempts have been made to produce vaccines to prevent metritis and preliminary
108 results are encouraging (Machado et al., 2014). However, after the resolution of clinical disease,
109 cows still have reduced pregnancy/ AI (Borsberry and Dobson, 1989; LeBlanc et al., 2002;
110 Kasimanickam et al., 2004; Sheldon et al., 2009). The mechanistic reasons behind continued
111 infertility following resolution of infection (metritis), or resultant inflammation (endometritis),
112 remains to be elucidated.

113 PATHOGENIC AGENTS INVOLVED IN UTERINE INFECTION

114 Microbial contamination of the uterus occurs shortly after parturition by a number of
115 opportunistic bacteria including *Escherichia coli*, *Trueperella pyogenes*, and the anaerobes
116 *Prevotella sp*, *Fusobacterium necrophorum*, and *Fusobacterium nucleatum*. The first bacterial
117 pathogen to colonize the upper reproductive tract following parturition involved in uterine
118 disease is *E. coli* (Williams et al., 2007); and endometrial specific strain of *E. coli*, termed
119 endometrial pathogenic *E. coli* (**EnPEC**) are distinct from gastro-intestinal and extra-intestinal
120 pathogenic *E. coli* (Sheldon et al., 2010). Analysis of this specific strain revealed a surprising
121 lack of virulence factors associated with EnPEC compared with pathogenic enteric and extra-
122 intestinal pathogenic *E. coli*. However, EnPEC possess an increased ability to adhere and invade
123 endometrial cells than other *E. coli* strains. Characterization of other metritis associated bacteria,
124 including some *E. coli* strains, have shown specific virulence factor expression like FimH
125 (Sheldon et al., 2010; Bicalho et al., 2012). Endometrial pathogenic *E. coli* induce an
126 endometrial inflammatory response due to the presence of the cell wall component
127 lipopolysaccharide (**LPS**; i.e., an endotoxin). Endometrial specific *E. coli* have now been
128 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-1 β , IL-6, IL-8 and PGF_{2 α} (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

152 infection resulting in clinical signs of endometritis with a purulent discharge in the uterus and
153 vagina (Amos et al., 2014; Goldstone et al., 2014a).

154 The above mentioned microbes dominate the literature regarding uterine disease, but it is
155 important to consider the presence of lesser studied bacterial species associated with disease. The
156 anaerobes *Prevotella* sp, *F. necrophorum*, and *F. nucleatum* have all been associated with severe
157 uterine disease in cattle and appear to aid the pathogenesis of both *E. coli* and *T. pyogenes*
158 (Olson et al., 1984). For example, *F. necrophorum* produces a leukotoxin which inactivates and
159 kills leukocytes required to clear an infection (Narayanan et al., 2002). *Prevotella*
160 *melaninogenica* has been shown to produce substances which inhibit phagocytosis of bacteria
161 and induce the production of factors by the host immune system to cause tissue destruction
162 (Jones and Gemmell, 1982; McGregor et al., 1986). It is interesting to note that other bacterial
163 strains are also present in the uterus and are not associated with uterine disease, such as
164 *Staphylococci* and *Streptococci* (Williams et al., 2005). Furthermore, a wide variety of microbes
165 can be identified in the postpartum uterus by molecular techniques, although their roles remain
166 unclear (Machado et al., 2012).

167 **TOLL-LIKE RECEPTOR SIGNALING**

168 Bacteria utilize specialized virulence factors to cause tissue damage and promote disease,
169 which leads a host response to these bacteria directed by the innate immune system, including
170 antimicrobial peptides, complement, and the Toll-like receptor (**TLR**) family. It is known that
171 antimicrobial peptides and complement play a critical role in the initiation of inflammation and
172 clearance of microbes from the uterus of cows (Bondurant, 1999); however, this review will
173 focus on the role of TLR. Since Hoffman and Beutler identified the importance of Toll in flies
174 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-1 β , 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

220 reduced circulating estradiol and perturbed prostaglandin signaling resulting in disruption of
221 ovarian cyclicity, extended luteal phases, delayed ovulation, slower follicle growth and increased
222 risk of anovulation (Opsomer et al., 2000; Sheldon et al., 2002; Herath et al., 2007). The changes
223 in follicular estradiol production are a direct result of reduced aromatase activity in granulosa
224 cells due to LPS exposure (Price et al., 2013; Magata et al., 2014). Furthermore, LPS induces a
225 switch of endometrial PG production from luteolytic PGF_{2α} to immune modulatory PGE₂ that
226 likely contributes to the extension of the luteal phase (Herath et al., 2009a).

227 ***Endometrial Response to Uterine Infection***

228 Uterine responsiveness to invading microbial pathogens must be rapid and robust to
229 prevent the establishment of uterine infection. Pro-inflammatory genes such as *IL-1α*, *IL-1β*, *IL-*
230 *6*, *TNFα* and *PTGES* have been shown to be upregulated in the endometrium of animals with
231 persistent endometritis compared to healthy cows (Herath et al., 2009b; Wathes et al., 2009;
232 Fischer et al., 2010). These pro-inflammatory agents increase recruitment of neutrophils and
233 macrophages to combat infection and aid in resolution (Sheldon et al., 2010). The S100 proteins
234 have recently been described to contribute to the inflammatory function of neutrophils,
235 macrophages, and mast cells (Goyette and Geczy, 2011). Endometrial expression of S100A8,
236 S100A9 and S100A12 rapidly increases in response to the inflammatory mediators IL-6 and IL-
237 10 induced by infection (Swangchan-Uthai et al., 2012). In addition, there is increased liver
238 production of serum amyloid A and haptoglobin in cows with uterine disease after parturition
239 (Sheldon et al., 2001). In vitro studies using endometrial explants, or purified endometrial
240 epithelial and stromal cells have shown similar inflammatory responses to *E. coli*, *T. pyogenes*
241 and highly purified bacterial cell wall components, LPS, lipoprotein and peptidoglycan (Borges
242 et al., 2012; Amos et al., 2014; Turner et al., 2014). Induction of proinflammatory mediators IL-

243 1β , IL-6 and IL-8 have all been shown to occur in these tissues in a TLR dependent manner
244 depending on the bacterial components utilized (Herath et al., 2006; Cronin et al., 2012; Turner
245 et al., 2014). In combination, increased inflammatory mediators, cellular influx of immune cells
246 and induction of antimicrobial factors all work in concert to combat and clear the active uterine
247 infection. However, as we have alluded to, this response may contribute to the infertility
248 witnessed in cows following the resolution of infection. Indeed, when assessing endometrial
249 expression of the inflammatory mediators *IL-1 α* and *IL-1 β* in cows with infection which became
250 infertile, both mediators are expressed at higher abundance in infertile cows compared with those
251 which remained fertile (Herath et al., 2009b). It is conceivable that an unchecked, excessive
252 endometrial inflammatory response could contribute to infertility in cows following infection.
253 One obvious mechanisms is disruption of the pre-implantation developmental environment.
254 Many embryotrophic factors produced by the oviduct and endometrium are also immune
255 modulators, and as such excessive or inappropriate temporal expression of these factors may
256 perturb embryonic development and negatively impact fertility. Indeed when embryos are
257 cultured in the presence of endometrial fluid from an inflamed uterus, total blasomere number
258 and allocation are negatively impacted (Hill and Gilbert, 2008). Similarly one could surmise that
259 embryo attachment and\or placentation could be equally effected by an inappropriately inflamed
260 uterine environment.

261 ***Ovarian Response to Uterine Infection***

262 When considering the mechanisms of infertility following uterine infection, the ovary is a
263 logical target as it contains a vulnerable and finite reserve of oocytes required for subsequent
264 generations; but how does infection at a distant site impact ovarian tissues? As described above,
265 ovarian function is perturbed following infection with reduced estradiol production, delayed

266 ovulation, retarded follicle growth, and extended luteal phases. Beyond the direct effects of
267 endocrine dysregulation on the ovary by altered LH patterns and shifts from endometrial PGF_{2α}
268 to PGE₂ synthesis, cellular and molecular pathways within the ovarian follicle are also affected
269 in cows suffering uterine infection. Key to the changes seen in the ovary is the presence of LPS
270 within the follicular fluid of diseased cows (Herath et al., 2007). We are now beginning to
271 understand the mechanisms by which the follicular environment, which is free of immune cells,
272 can contribute to infertility in dairy cows.

273 Granulosa cells possess the molecular machinery required for detection of bacterial
274 components, TLR, CD14 and MD-2. In addition, granulosa cells exposed to the bacterial
275 components LPS or peptidoglycan mount an acute inflammatory response by increased
276 production of inflammatory mediators IL-1β, IL-6, IL-8 and TNFα (Herath et al., 2007;
277 Bromfield and Sheldon, 2011; Price et al., 2013; Price and Sheldon, 2013). The granulosa cell
278 inflammatory response to LPS is initiated by TLR4 and intracellular signaling occurs through
279 rapid phosphorylation of the extracellular-signal-regulated kinases (**ERK**) and p38 kinase
280 pathways (Bromfield and Sheldon, 2011; Price et al., 2013). Estradiol production by granulosa
281 cells is reduced following LPS exposure by reducing aromatase expression, however the
282 mechanism of aromatase reduction is unclear (Herath et al., 2007). Of paramount interest is the
283 finding that oocyte maturation is also perturbed in the presence of LPS (Bromfield and Sheldon,
284 2011).

285 Oocyte maturation occurs spontaneously *in vitro*, developing from the germinal vesicle
286 stage to the metaphase II (**MII**) stage in approximately 24 h. This highly orchestrated
287 development matures both the nuclear and cytoplasmic compartments of the oocyte for the first
288 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 hyaluronan, 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, TNF α , 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

312 intracellular signaling pathways utilized by these various signaling moieties uses the central
313 phosphatidylinositol-4,5-bisphosphate 3-kinase (**PI3K**)/ protein kinase B (**AKT**) pathway critical
314 for oocyte maturation (Okumura et al., 2002; Van Slyke et al., 2005). It is currently unclear
315 whether the presence of bacterial pathogens alters these intracellular pathways in oocytes,
316 reducing developmental competence of oocytes by disrupting cytoplasmic or nuclear maturation.

317 The negative effects of LPS on developing oocytes in the dominant follicle explains
318 infertility shortly after infection, but how is long-term infertility explained in animals following
319 uterine infection? We propose that infection also perturbs smaller, developing follicles including
320 primordial stage follicles. Initiation of folliculogenesis is a tightly orchestrated series of
321 molecular and cellular events. Primordial follicles containing an immature oocyte at the
322 dictyotene stage of meiosis are held in a quiescent state by the presence of inhibitor factors
323 including phosphatase and tensin homolog (**PTEN**) and forkhead box O3a (**FOXO3a**)
324 (Castrillon et al., 2003; Reddy et al., 2008; Bao et al., 2011). In vitro culture of cortical ovarian
325 explant results in the unexplained spontaneous activation of the primordial follicle pool to
326 develop primary and secondary follicles. In the presence of LPS primordial follicle activation is
327 increased, resulting in an enlarged pool of primary follicles and a depletion of the primordial
328 follicle reserve (Bromfield and Sheldon, 2013). *In vivo* studies using mice revealed a similar
329 decrease in the primordial follicle reserve in conjunction with an increase in follicle atresia after
330 administration of LPS. The LPS induced activation of the primordial pool occurs in conjunction
331 with loss of PTEN and FOXO3a protein in primordial follicles, but it is unclear if this is
332 causative or resultant of follicle activation. Ovarian explant cultures increase production of
333 inflammatory mediators IL-1 β , IL-6 and IL-8 in response to LPS. It was interesting to note the
334 high basal level of IL-6 production in ovarian explants cultured in control medium, which we

335 propose is involved in the spontaneous activation of primordial follicles. Assessment of larger
336 pre-antral follicle responses to LPS reveal that these stages, at least in vitro, appear to be resistant
337 to the effects of LPS, with no change in estradiol production, or oocyte and follicle growth
338 (Bromfield and Sheldon, 2013). Although the above studies investigate the role of LPS on
339 follicle growth *in vitro*, they suggest that fertility may be affected in both short and long term
340 scenarios with the primordial follicle pool inappropriately activated impacting long-term fertility,
341 while low quality oocytes from dominate follicles may impact short-term fertility. The precise
342 mechanisms by which LPS exposure reduces the primordial follicle pool and oocyte
343 development remain to be elucidated, but we propose that alterations in redundant signaling
344 pathways integral in both immunity and development could play a major role. As in oocyte
345 development, the PI3K/AKT pathway is critical for coordinated recruitment of primordial
346 follicles (Wandji et al., 1996; Fortune, 2003). Similarly, activation of the TLR signaling also
347 uses the central PI3K/AKT pathway to increase production of inflammatory mediators like IL-6,
348 feeding back into the pathway for increased stimulation (Laird et al., 2009). We propose that
349 activation of the TLR4/IL-6 by bacterial pathogens contributes to the inappropriate recruitment
350 of primordial follicles by activation of the same pathway (**Figure 2**). To explore the links
351 between uterine disease and the ovary will require exploiting animal models.

352 **ANIMAL MODELS OF UTERINE INFECTION AND THEIR IMPLICATIONS**

353 In 1929, Nobel laureate August Krogh coined the principle that “for such a large number
354 of problems there will be some animal of choice on which it can be most conveniently studied.”
355 (Krogh, 1929; Albertini, 2011). Krogh’s principle encompasses the development of PCR, using
356 thermo stable Tap polymerase from heat labile bacteria (Chien et al., 1976); the study of
357 menstruation in the short tailed fruit bat (Rasweiler and de Bonilla, 1992); and the utilization of

358 jellyfish green fluorescent protein in cell biology (Chalfie et al., 1994). Unique to animal science,
359 we have ready access to the animals in which the problem is relevant. However, uterine infection
360 in dairy cows shares a number of similarities to puerperal fever and pelvic inflammatory disease
361 (**PID**) in women. Women who suffer PID, do so as a result of uterine infection usually bought on
362 by sexually transmitted bacterial infections such as *Gonorrhea* and *Chlamydia* (Ross, 2002).
363 Pelvic inflammatory disease causes pain and infertility, and is the leading cause of gynecological
364 hospitalization of women in the developed world (Ross, 2002). Studies in women suggest that
365 PID causes ovarian changes similar to those seen in the dairy cow following uterine infection
366 (Weiner and Wallach, 1974; Margolis, 1976; Bychkov, 1990). In addition, studies of infection
367 and immunity using primary cells from the bovine uterus and ovary are similar to studies using
368 human endometrial and ovarian cells (Sanchotello et al., 1992; Allhorn et al., 2008; Price et al.,
369 2012). The similarities between human PID and bovine uterine disease give us a unique
370 opportunity to understand a disease state pertinent to both agricultural production and human
371 health. For humans, the impact of uterine disease might be particularly important for patients
372 who have unexplained infertility and/or a need for assisted reproduction techniques such as in
373 vitro fertilization where the immune environment requires a balance between physiological (for
374 normally ovarian function) and pathological (to combat bacterial infection) response (Chegini et
375 al., 2002; Li et al., 2006). The availability of bovine oocytes and granulosa cells may provide an
376 opportunity to inform human studies relevant to puerperal fever and PID in women.

377 Several animal models of uterine infection have been developed with varying degrees of
378 success in reproducing the disease. The mouse has been commonly used by infusing bacteria into
379 the uterus. We have used the mouse as a convenient model by administering LPS
380 intraperitoneally and noted that while primordial depletion occurs, it is likely due to follicle

381 atresia as opposed to increased primordial follicle activation as in the cow (Bromfield and
382 Sheldon, 2013). In the dairy cow it has long been established that the endocrine status of the
383 animal is key to the development and severity of uterine infection following infusion of *E. coli*
384 and *T. pyogenes*. Induction of the disease at estrus or administration of exogenous estradiol limits
385 the formation of infection, whereas bacterial infusion during the luteal phase or exogenous
386 progesterone administration increases the likelihood of uterine infection persisting (Rowson et
387 al., 1953; Ayliffe and Noakes, 1982). In addition, the structural integrity of the endometrium is
388 important, as noted above physical damage to the epithelial layer seems to be important in
389 establishing disease. Uterine infusion of the *T. pyogenes* virulence factor, PLO without
390 endometrial damage results in no signs of disease (Miller et al., 2007). However, uterine infusion
391 of *T. pyogenes* with mechanical disruption of the endometrium results in uterine disease (Amos
392 et al., 2014). The potential to exploit the dairy cow model of uterine disease has yet to be fully
393 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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737

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

745

746

FIGURE CAPTIONS

747 **Figure 1.** Schematic representation of uterine infection and impacts on the reproductive tract.
748 This figure represents the all-encompassing effects of uterine bacterial infection on
749 neuroendocrine signaling, uterine health and ovarian function. Brain; GnRH and LH production
750 are reduced. Endometrium; bacterial pyolysin (**PLO**) disrupts endometrial cells by osmotic lysis,
751 while lipopolysaccharides (**LPS**) initiates an inflammatory response via Toll-like receptor (**TLR**)
752 4 activation increasing cytokine, chemokine and PGE₂ production. Ovary; the primordial follicle
753 reserve is depleted, follicle growth is retarded and luteal phase prolonged. Ovarian granulosa
754 cells respond to bacterial LPS in a TLR4 dependent manner increasing inflammatory mediators,
755 reducing aromatase and estradiol, and reducing oocyte competence. Illustration by Stacey Jones,
756 UF/IFAS.

757 **Figure 2.** Schematic representation of the redundancies between Toll-like receptor (**TLR**) 4/IL-
758 6 signaling and the cellular pathways in granulosa cells and oocytes involved in primordial
759 follicle activation. The left panel shows the intracellular pathways of TLR4 or IL-6 activation
760 through phosphatidylinositol-4,5-bisphosphate 3-kinase (**PI3K**) and Protein kinase B (AKT).
761 The right panel shows the process of primordial follicle activation utilizing the same PI3K and
762 AKT pathway which is regulated by the balance of phosphatase and tensin homolog (**PTEN**)
763 activation by tyrosine kinase receptors. We propose that bacterial activation of the TLR/IL-6
764 pathway (left) contributes to inappropriate activation of the follicle activation pathway (right).

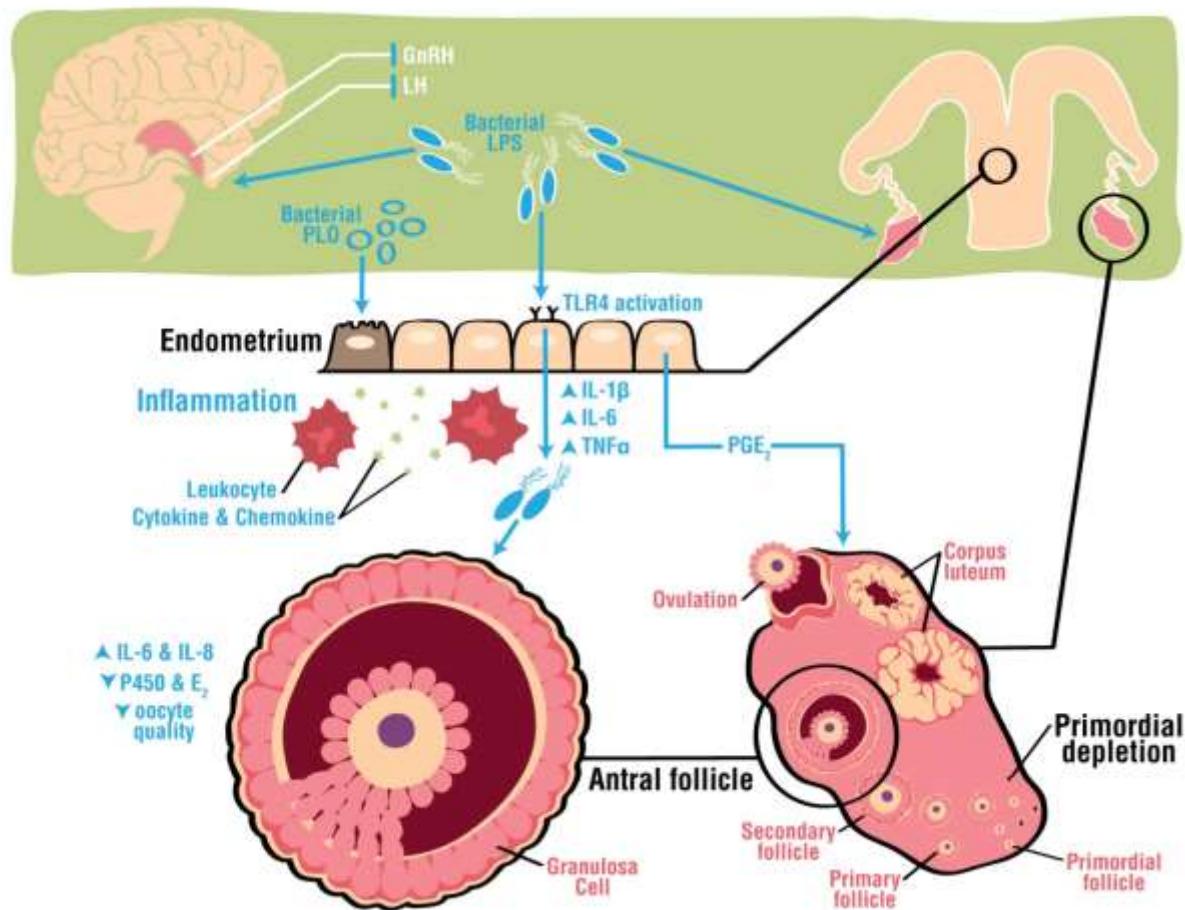
Figure 1

Figure 2

