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Recent advances in the immunology and uterine microbiology of healthy cows and cows that develop uterine disease

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Abstract: Uterine diseases are highly prevalent in dairy cows. Causes of uterine diseases are multifactorial. There is good evidence for the susceptibility of the host and for the role of pathogenic bacteria, and less evidence for the effect of the environment. Uterine and leukocyte immune response is impaired early postpartum in cows that develop uterine disease. The decrease in immune function is associated with a decrease in calcium postpartum and an increase in NEFA and BHBA. Both endometrial cells and granulosal cells possess toll-like receptors (TLR) that can recognize and mount an inflammatory response to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPSs) or lipopeptides. Exposure to LPSs leads to endocrine dysregulation, which may affect steroidogenesis, ovulation and luteolysis. Some *E. coli* possess specific virulence factors such as fimH, hlyA, cdt, kpsMII, ibeA, and astA that cause uterine disease in dairy cows. These *E. coli* are associated with the occurrence of other pathogenic bacteria such as *A. pyogenes*, *F. necrophorum*, and *Bacteroides* spp., which act synergistically to cause uterine disease. The combined effect of bacterial infection and activation of inflammation is damage to the endometrium and embryo, delayed ovulation, shortened or extended luteal phase, and decreased fertility.

Key words: Immune function, uterine pathogens, uterine diseases, dairy cows

1. Introduction

The transition to lactation (3 weeks before to 3 weeks after calving) is a challenging period for a high producing dairy cow. This period is characterized by a sharp decrease in immune function (1–3). At the same time, physical barriers such as the cervix are breached at parturition, which allows rapid colonization of the uterus by bacteria (4). The immune system needs to recognize and eliminate pathogenic bacteria from the uterus in order to prevent disease. Nonetheless, with the decrease in immune function and the large bacterial challenge, the system is overwhelmed, and uterine diseases such as metritis, clinical endometritis, and subclinical endometritis are established in a large proportion of cows in early postpartum. Metritis affects about 20% of lactating dairy cows, with the incidence ranging from 8% to >40% in some farms (5–8). Clinical endometritis also affects about 20.0% of lactating dairy cows, with the prevalence ranging from 5% to >30% in some herds (8–10). Subclinical endometritis is the most prevalent of all uterine diseases; it affects ~30% of lactating dairy cows, with the prevalence ranging from 11% to >70% in some herds (8,11,12). These diseases have been associated with decreased pregnancy per artificial insemination (AI), extended interval to pregnancy, increased culling, and economic losses (4,12,13).

The causes of uterine disease are multifactorial. Of the 3 components of the disease triangle, there is good evidence for the susceptibility of the host and for the role of pathogenic bacteria. There is less evidence for the effect of the environment (4), although it cannot be disregarded (14). Therefore, this review will focus on the effect of systemic and cellular indicators of energy balance on immune function and the effect of calcium status on immune function and susceptibility to uterine diseases, the mechanism of recognition of pathogens by professional phagocytes, the uterine endometrium and granulosal cells, the main pathogens that cause uterine disease, and the immune response to pathogens by the uterine endometrium and professional phagocytes.

2. The role of glucose and Ca^{2+} on neutrophil function and innate immunity

Neutrophils are the main leukocyte cells involved in clearing bacteria after uterine infection (15); however,

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during the transition into lactation, dairy cows experience a reduction in neutrophil function, including reduced phagocytosis and killing capacity (1,3,16). The transition into lactation is a period of energy and mineral deficiency in which cows have to rely on their energy stores for their normal functions (17–19). This period is characterized by a decrease in dry-matter intake (DMI), leading to a sharp decrease in glucose and minerals, especially calcium, and an increase in body fat mobilization in the form of nonsterified fatty acids (NEFAs) to meet the energy demands for maintenance, growth, and milk production, which results in accumulation in the liver of products of incomplete oxidation of NEFAs, such as beta-hydroxybutyrate (BHBA) (17–19). Therefore, innate immunity may be negatively affected by the limited supply of glucose and calcium required for neutrophil activation, chemotaxis, phagocytosis, and oxidative burst or by the immunosuppressive effects of fatty acids and their metabolites on immune cells. Concentrations of BHBA similar to those of cows with subclinical ketosis impaired neutrophil phagocytosis, extracellular trap formation, and killing of bacteria (20,21). Others have shown that addition of NEFAs to the culture medium affected proliferation of peripheral blood mononuclear cells and oxidative burst of neutrophils (22). The mechanism by which NEFAs and BHBA affect the immune system has not been elucidated. Nonetheless, recent research has found that BHBA is a main ligand for the nicotinic acid (Niacin) receptors HM74A (GPR109a) and HM74 (GPR109b) (23,24), and that activation of these receptors, especially HM74A, has widespread anti-inflammatory effects including reduction in leukocyte migration and generation of reactive oxygen species, which has been shown to be beneficial for prevention of atherosclerosis and cardiac disease (25,26) but may be a predisposing factor to uterine disease if a proper inflammatory response is not mounted.

Neutrophils rely on hexose carbon to generate ATP through glycolytic pathways. Neutrophils depend mainly on extracellular glucose to generate ATP for chemotaxis, but they also can store and use glycogen when supply of extracellular glucose is limited. On the other hand, neutrophils depend primarily on intracellular glycogen to generate glucose for phagocytosis and intracellular killing of pathogens (27–29). Chemotactic stimuli accelerated glucose uptake by neutrophils, whereas phagocytic stimuli by opsonized zymosan particles failed to increase glucose uptake, but increased glycogenolysis (28,29). Therefore, the decline in blood glucose in early lactation, observed in cows suffering from more severe negative energy balance, might impair neutrophil chemotaxis and could lead to decreased cytosolic glycogen stores, which might lead to suppressed cell function and predispose cows to disease. We recently demonstrated that neutrophil glycogen content was reduced in cows developing metritis compared with healthy cows on the day of calving and at 7 and 42 days postpartum. Cows with subclinical endometritis (SCE) had lower PMN glycogen content than healthy cows at 7, 28, and 42 days postpartum (Figure 1) (30). The key observation was that a decrease in neutrophil glycogen stores was observed before the development of the disease (metritis was diagnosed at 7 ± 4 days postpartum and SCE at 42 days postpartum), which indicates that low neutrophil glycogen is a risk factor for the development of uterine diseases. Furthermore, once the disease is established, glucose metabolism and glycogenesis may be affected. Exposure to bacterial LPS has been found to affect both glucose and glycogen synthesis by decreasing the activity of phosphoenolpyruvate carboxykinase, which is involved in gluconeogenesis (31), and glycogen synthase, which is the primary enzyme in glycogenesis (32). Pro-inflammatory cytokines also have been shown to alter glycogen metabolism. In rat hepatocytes, IL-6 and IL-1β lessened or completely abolished the increase of glycogen deposition when cells were stimulated with insulin (33,34). These cytokines inhibited activity of glycogen synthase and stimulated glycogen phosphorylase activity, thereby attenuating the effects of insulin on these enzymes. Kanemaki et al. (1998) demonstrated that IL-6 was very effective, as it decreased glycogen increase by 30% within 1 h and nearly abolished its increase within 4 h of cytokine treatment, whereas IL-1β showed no significant effects until 4 h (34).

Recent work at the University of Florida (Martinez et al., 2012) demonstrated that cows with subclinical hypocalcemia have neutrophils in blood that are less

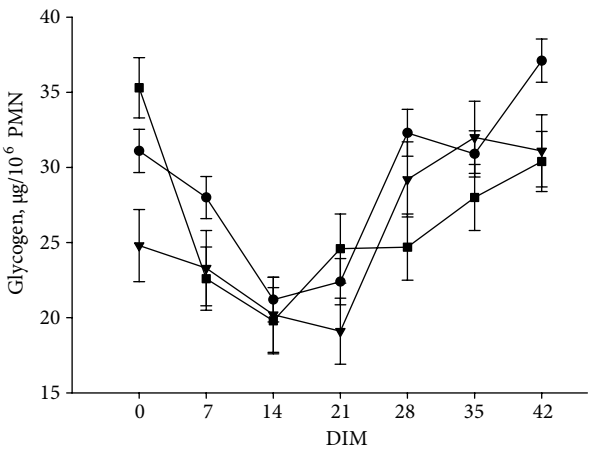

Figure 1. Least-squares means ± S.E.M. for blood PMN glycogen concentrations for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares), or remained healthy up to 42 DIM (circles). Adapted from Galvão et al. (30).

capable of phagocytizing and killing pathogenic bacteria in vitro (35,36). It was suggested that neutrophil function in cows with subclinical hypocalcemia is compromised by reducing cytosolic ionized calcium (Ca^{2+}) required for initiation of phagocytosis, although this process is not exclusively Ca-dependent (37). In addition, Ca^{2+} is necessary to control the fusion of secondary granules with the phagosomal membrane (38) during the bactericidal activity. Inadequate concentrations of Ca^{2+} in blood are likely to influence the availability for cellular function. Blood mononuclear cell cytosolic Ca^{2+} was reduced around parturition, and the reduction was greater in cows with hypocalcemia compared with those that were capable of restoring blood calcium concentrations quickly after calving (39). Neutrophil activation involves the binding of soluble inflammatory mediators to receptors on the cell neutrophil surface, followed by activation of cytosolic components such as phospholipase C, protein kinase C, and inositol 1,4,5-triphosphate. This transduction mechanism releases Ca^{2+} from the endoplasmic reticulum to increase cytosolic Ca^{2+} up to 10-fold its basal concentration (40). An adequate cytosolic Ca^{2+} is critical for activation of NADPH oxidase to produce reactive oxygen species (ROS) to effectively kill phagocytized pathogens (40). Moreover, once Ca^{2+} is released from the endoplasmic reticulum, receptors localized in the endoplasmic reticulum signal the plasma membrane to open Ca^{2+} membrane channels in a retrograde process called store-operated Ca^{2+} entry. This additional Ca^{2+} entry from the extracellular space helps replenish Ca^{2+} stores in endoplasmic reticulum (41). Based on previous work, it is hypothesized that cows with subclinical hypocalcemia have less endoplasmic reticulum Ca^{2+} to increase cytosolic concentrations and are unable to replenish the intracellular Ca^{2+} because of the reduced concentrations in blood (39,42). Impairing the rise in cytosolic Ca^{2+} reduces activation of neutrophils and generation of ROS, which could be reflected in decreased phagocytosis and killing activities. In a recent study at the University of Florida, it was observed that inducing subclinical hypocalcemia compromised leukocyte function (36), and spontaneous subclinical hypocalcemia resulted in an increased incidence of metritis (35). In fact, the probability of metritis markedly increased as serum Ca concentrations decreased in the first 3 days postpartum (Figure 2) (35). A 1 mg/dL decline in serum Ca between calving and the lowest value in the first 3 days postpartum increased the risk of metritis in 28% (adjusted risk ratio = 1.28; 95% CI = 1.10 to 1.49) (35). Therefore, it seems that a major component of the underlying mechanism for development of metritis in dairy cows is the inadequate concentrations of Ca^{2+} and glucose (hence glycogen) in early lactation that compromise immune function and allow utero-pathogenic bacteria to thrive in the uterus, thereby causing disease.

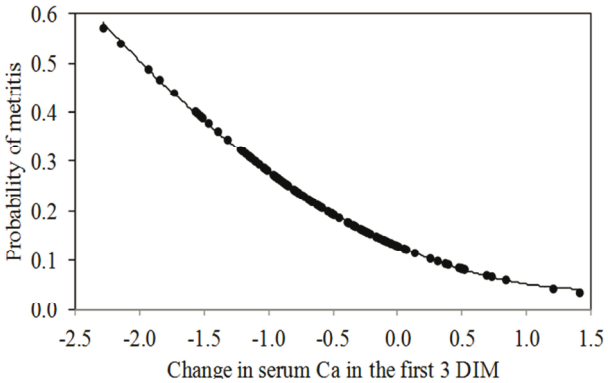

Figure 2. Probability of metritis relative to the change in serum Ca concentrations between the day of calving and the lowest serum Ca concentration within the first 3 days postpartum. Adapted from Martinez et al. (35).

3. Mechanism of recognition of pathogens by professional phagocytes and epithelial cells and the process of mounting an immune response

Sentinel cells such as dendritic cells, phagocytes such as macrophages and neutrophils, and certain epithelial cells such as intestinal epithelial cells recognize pathogen-associated molecular patterns (PAMPs) present in microbial invaders through pattern-recognition receptors (PRRs). Examples of PAMPs include lipopolysaccharide from gram-negative bacteria, lipoteichoic acid, peptidoglycan from gram-positive bacteria, and zymosan from yeast (43,44). The most important group of PRRs is the toll-like receptor (TLR) family. There are 10 well characterized and widely expressed TLRs in mammals (45); TLR1, TLR2, and TLR6 recognize lipids found in all bacteria and fungi; TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids from viruses and bacteria; TLR4 recognizes lipopolysaccharide (LPS) and polysaccharides found in gram-negative bacteria, fungi (mannan), and parasites (glycoinositolphospholipids from Trypanosoma); TLR5 recognizes flagellin in flagellated bacteria; and TLR10 still has no recognized ligand (45). Triacylated lipopeptides are the most common type of lipopeptide in gram-negative bacteria and bind TLR2, which heterodimerizes with TLR1 in mice, whereas diacylated lipopeptides are found in gram-positive bacteria or mycoplasma and bind TLR2/TLR6 heterodimers (45). After contact with bacteria through TLRs, leukocytes or epithelial cells are stimulated to produce and release pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin (IL) -1, IL-6, and chemokines such as IL-8 and monocyte chemoattractant protein 1 (MCP-1) (46,47). Later in the inflammatory process, anti-inflammatory cytokines (e.g., IL-10) are released to minimize the deleterious effects of chronic inflammation (46). Pro-inflammatory cytokines

and chemokines induce neutrophil and monocyte diapedesis and chemoattraction to the site of infection to phagocytize and kill invading pathogens. Professional phagocytes kill pathogens by production of ROS, release of proteolytic granules (48), and the formation of extracellular traps (49). The last method is particular to neutrophils. The release of proteolytic enzymes also may cause collateral damage to host cells.

While neutrophils are the main phagocytic leukocytes, monocytes and macrophages are actively involved in immunomodulation after infection. We recently evaluated the cytokine expression by blood monocytes of lactating Holstein cows with or without postpartum uterine disease (50). Relative to unstimulated cells, *E. coli*-stimulated monocytes from cows with metritis had lower gene expression of key pro-inflammatory cytokines than monocytes from healthy cows from calving to 14 days after calving (TNFα at 0, 7, and 14 days after calving, IL-1β and IL-6 at 7 and 14 days after calving; Figure 3). Furthermore, concentration of TNFα was lower in the culture medium of *E. coli*-stimulated monocytes from cows with metritis than from healthy cows at calving and 7 and 21 days

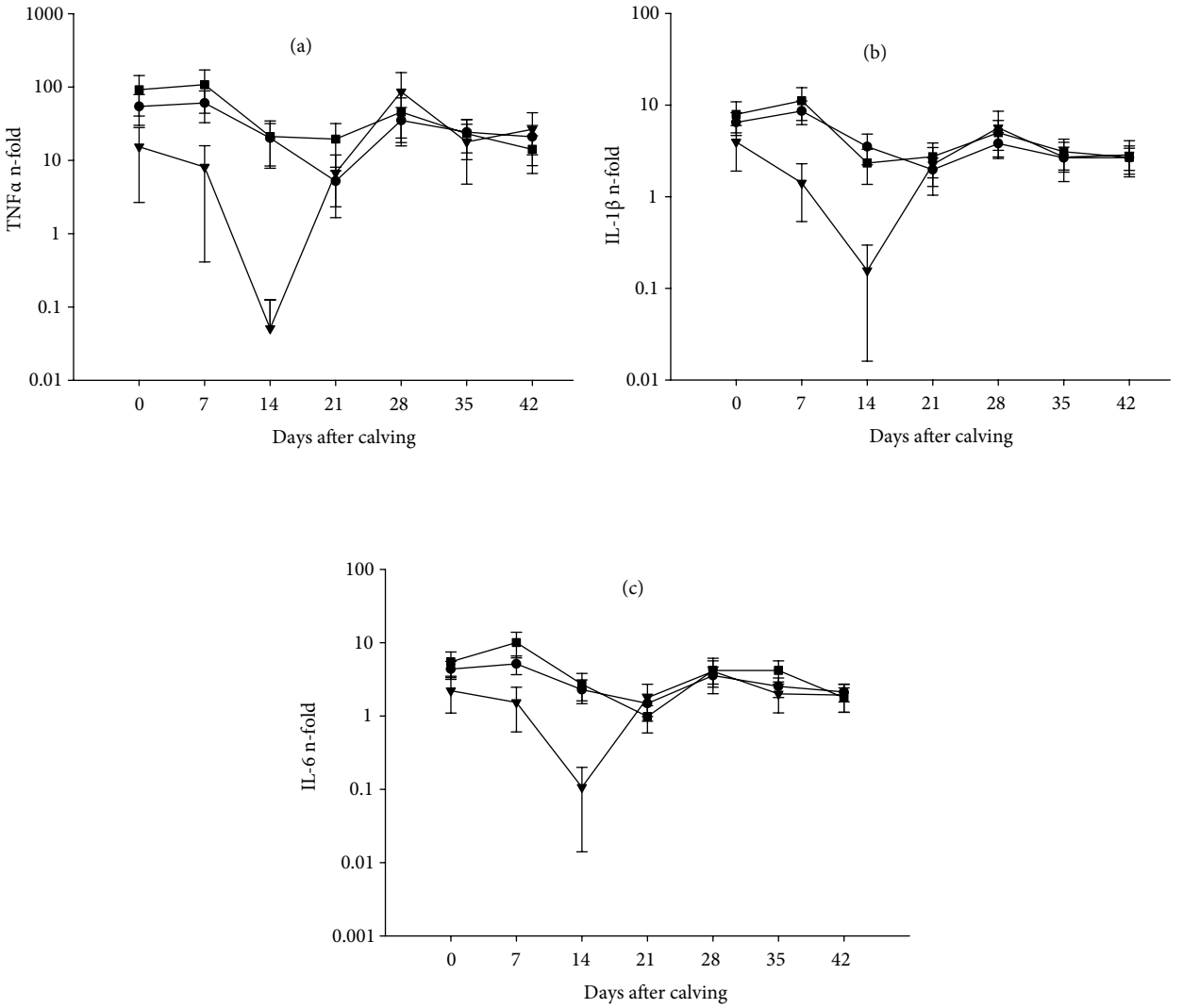

Figure 3. Fold change in mRNA expression in *E. coli*-stimulated cells in relation to nonstimulated cells for TNFα (a), IL-1β (b), and IL-6 (c) respectively, in cows that developed metritis up to 14 days after calving (triangles), cows that had endometritis at 42 days after calving (squares), or cows that remained healthy up to 42 days after calving (circles). Metritis was characterized by fetid uterine discharge and fever (≥39.5 °C). Endometritis was characterized by presence of ≥10% neutrophils in uterine cytology. Healthy controls did not have metritis or endometritis up to 42 days after calving. Cows that developed metritis had decreased ($P \leq 0.05$) TNFα gene expression at 0, 7, and 14 days after calving, and decreased IL-1β and IL-6 at 7 and 14 days after calving compared to cows that had endometritis and control cows. Adapted from Galvão et al. (105).

after calving. We concluded that altered expression and production of pro-inflammatory cytokines postpartum could contribute to impaired inflammatory response and predispose cows to development of metritis.

4. Identification of TLRs in the uterine endometrium and the endometrial inflammatory response in healthy cows and cows with uterine disease

In 2008, a group in the UK led by Dr Martin Sheldon characterized the presence of TLRs in the uterine endometrium (51). They observed that the endometrium expressed TLRs 1 to 10, whilst purified populations of epithelial cells expressed TLRs 1 to 7 and 9, and stromal cells expressed TLRs 1 to 4, 6, 7, 9, and 10. They also observed that TLRs appeared to be functional as epithelial cells secreted prostaglandin E2 in response to bacterial PAMPs from gram-negative and gram-positive bacteria such as LPS and lipoteichoic acid, respectively. In addition, they observed that epithelial cells expressed antimicrobial peptides, such as Tracheal and Lingual Antimicrobial Peptides (TAP and LAP) and Mucin-1, which were upregulated when the cells were treated with LPS. This was an important finding because it demonstrated that the uterine endometrium could recognize pathogens and help mount an immune response. Later, Dr Sheldon's group showed that TLR4 mediated the response of epithelial and stromal cells to LPS in the endometrium (52). They created mutant mice lacking TLR4 and showed that intrauterine infusion of purified LPS induced an inflammatory response with accumulation of granulocytes throughout the endometrium of wild type (WT) but not Tlr4(–/–) mice. Stromal and epithelial cells isolated from the endometrium of WT but not Tlr4(–/–) mice secreted IL-6, the chemokines CXCL1 and CCL20, and prostaglandin E_2 (PGE_2) in response to LPS. They later showed that lipopeptides found in gram-positive and gram-negative bacteria also stimulated an inflammatory response by epithelial and stromal cells of bovine endometrium via TLR2, TLR1, and TLR6 (53). These data definitely confirm that the bovine endometrium participates in mounting an inflammatory response to uterine pathogens. In another study, Dr Sheldon's group showed that bacterial LPS induced an endocrine switch from prostaglandin $F_{2\alpha}$ alpha ($PGF_{2\alpha}$) to PGE_2 in bovine endometrium (54). This was an important finding because a decrease in $PGF_{2\alpha}$ and an increase in PGE_2 could interfere with luteolysis. Earlier studies had observed greater concentrations of both $PGF_{2\alpha}$ and PGE_2 but also a greater ratio of PGE_2 to $PGF_{2\alpha}$ in the uterine lumen of cows with pyometra (cows with pyometra have a persistent corpus luteum), which suggests that the ratio of PGE_2 to $PGF_{2\alpha}$ might be more important for luteolysis than the absolute concentrations of both hormones (55). Furthermore, IL-1 and IL-6 decreased the expression of oxytocin receptors in endometrial cells, which could also impair the mechanism of luteolysis (56). Indeed, one of the observed clinical effects of endometritis is a delay or lack of luteolysis (57,58). On the other hand, the pro-inflammatory cytokine TNFα and interferons have been found to stimulate the release of $PGF_{2\alpha}$ from the endometrium and luteal cells and to induce luteolysis (59–62). Therefore, inflammation could have a bimodal effect on the length of the estrous cycle, whereby it could induce luteolysis in some cows and delay luteolysis in others. The fate of the corpus luteum seems to be dependent on the degree of inflammation. A low concentration of TNF stimulated in vivo luteolytic factors such as $PGF_{2\alpha}$, leukotriene C4, and nitrous oxide (NO) as well as induced apoptosis, whereas the high concentration of TNF stimulated a survival pathway in the bovine corpus luteum increasing luteal content of progesterone (P4) and PGE_2 (63).

We have looked at the uterine inflammatory state in cows that remain healthy and cows that develop uterine disease by comparing the gene expression of important pro-inflammatory (TNFα, IL-1β, IL-6) and anti-inflammatory (IL-10) cytokines, and the main neutrophil chemokine (IL-8) from calving until week 7 after calving in cows that developed endometritis and healthy control cows (64). Endometritis was evaluated at week 5 by uterine lavage and cytology. Interestingly, 2 main pro-inflammatory cytokines (i.e. TNFα and IL-1) were decreased in cows with endometritis compared with control cows at calving or at week 1 while pro-inflammatory cytokines (i.e. IL-1 and IL-6) and the chemokine IL-8 were increased at weeks 5 or 7 (Figure 4). These data indicate that lower local expression of pro-inflammatory cytokines in the endometrium early after calving might impair activation of inflammation and clearance of bacteria and lead to the development of endometritis.

5. Identification of TLRs in granulosa cells and the endocrine effect of exposure to PAMPs

Uterine disease not only affects the uterus but also the ovaries. LPS from gram-negative bacteria such as *E. coli* is increased in the uterine fluid (65), plasma (54), and follicular fluid (66) when cows have uterine infection. LPS impaired the release of both gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) (67), and decreased aromatase activity (54), which ultimately may affect follicular growth and estradiol production (68,69), and decreased ovulation rate (67,70). Magata et al. observed that follicles with high concentrations of LPS (>0.5 EU/mL, n = 15) had lower concentrations of E2 and higher concentrations of P4 when compared to follicles with low concentrations of LPS (<0.5 EU/mL, n = 23) (71). Furthermore, in follicles with high concentrations of LPS,

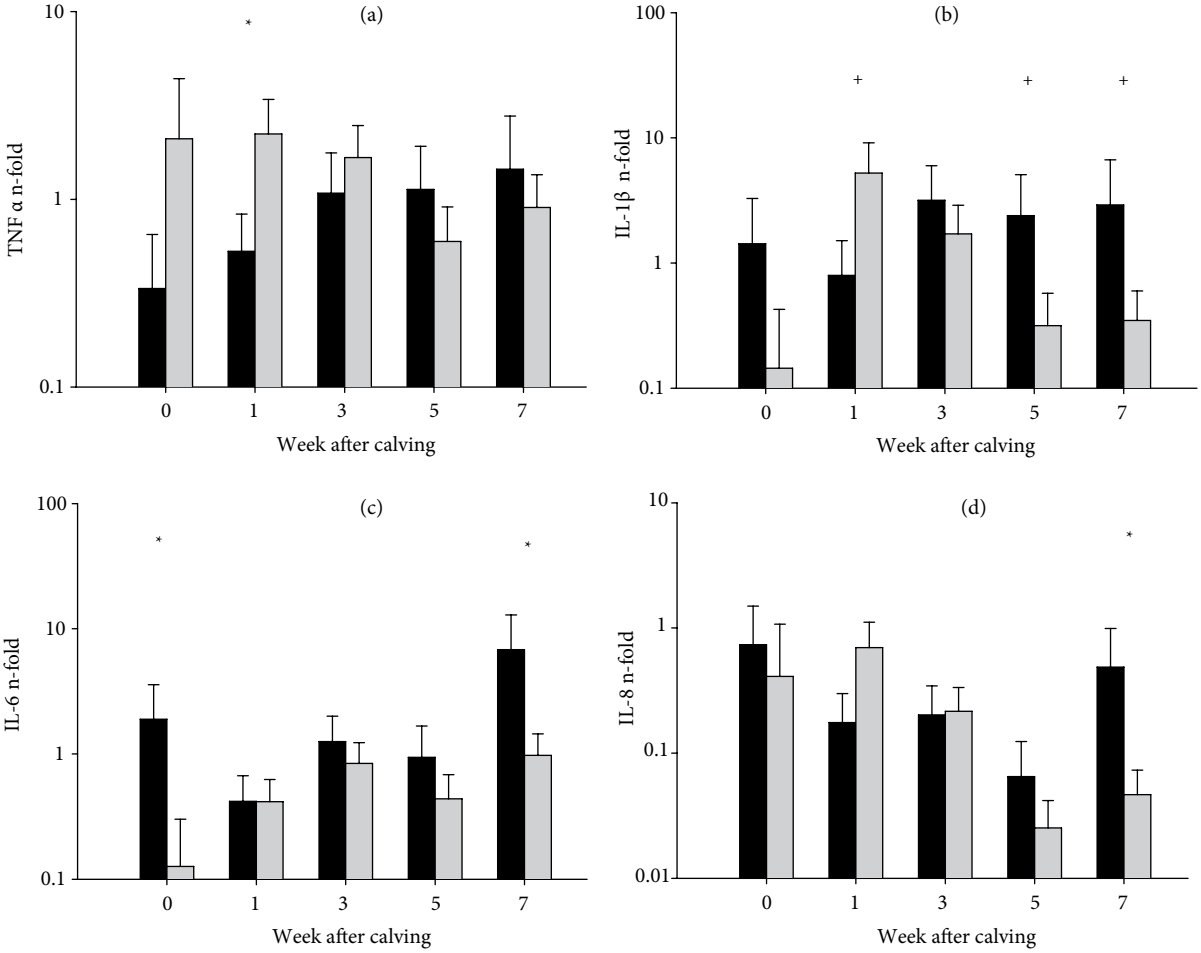

Figure 4. Fold change (n-fold) in TNFα (a), IL-1β (b), IL-6 (c), and IL-8 (d) mRNA gene expression in cows that had endometritis at week 5 (black bars; n = 11) and healthy control cows (gray bars; n = 17) from calving (0) until week 7 after calving. Endometritis was characterized by the presence of ≥10% neutrophils in the uterine cytology at week 5; control cows had <10% neutrophils in the uterine cytology. * $P \leq 0.05$ (significant differences); + $0.05 < P \leq 0.10$ (tendency towards statistical differences). Adapted from Galvão et al. (109).

transcripts of steroidogenic enzymes such as CYP17 and P450arom were lower. In those follicles, the expression of caspase-3 was high, indicating an association with follicular atresia (71). These findings indicate that LPS present in follicular fluid may cause ovarian dysfunction by inhibiting follicular activity. In a recent study evaluating risk factors for early ovulation postpartum in dairy cows, we observed that cows that had metritis and digestive problems such as diarrhea and displaced abomasum, that calved in the winter or spring, that had metabolic problems such as hypocalcemia or ketosis, or that lost >28 kg BW had decreased ovulation in the first 21 days postpartum (72). Recent work from the UK has observed that granulosal cells possess TLRs, and they can mount an inflammatory response via TLR2 and TLR4 (73,74). Price and Sheldon showed that granulosal cells from emerged bovine antral follicles expressed mRNA for all 10 TLRs, and cellular expression of mRNA for the cytokines IL1B, IL6, IL10, and TNF, and chemokines IL8 and CCL5, increased after treatment with synthetic bacterial lipoprotein binding TLR2, lipopolysaccharide binding TLR4, or flagellin binding TLR5. However, supernatants of granulosal cells accumulated IL-1beta, IL-6, and IL-8 protein in a concentration-dependent manner only when treated with lipoprotein or lipopolysaccharide, but not flagellin (74). In the work of Price et al., supernatants of primary bovine granulosal cells from dominant follicles accumulated IL-1β, IL-6, and IL-8 when treated for 24 h with Pam3CSK4 (PAM) that binds TLR2 or lipopolysaccharide (LPS) that binds TLR4. Granulosal cell responses to PAM or LPS were rapid, with increased phosphorylation of p38 and ERK1/2 within 30 min and increased abundance of IL6, IL1B, IL10, TNF, IL8, and CCL5 mRNA after 3 h of treatment. Furthermore, treatment with LPS or PAM reduced the

accumulation of estradiol and progesterone (73). In conclusion, bacterial PAMPs initiated inflammation and perturbed the endocrine function of bovine granulosal cells from emerged antral follicles and dominant follicles via TLR2 and TLR4 pathways.

6. Characterization of pathogenic bacteria that cause uterine disease

Virtually all cows are infected with bacteria in the days following calving (4). Bacterial culture of the postpartum uterus yields a wide range of isolates (75–80). A complete list of bacteria found using cultures has been described from samples of cows with metritis or endometritis (81), but mainly *E. coli*, *Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*), *Fusobacterium necrophorum*, and *Bacteroides* spp. have been identified in diseased cows, whereas *Streptococcus* spp., *Staphylococcus* spp., and *Bacillus* spp. have been identified in the uteri of healthy cows (77,79,82). Interestingly, metagenomic analysis of the uterine microbiota in healthy cows and cows with uterine disease (metritis and endometritis) confirmed that *F. necrophorum* and *Bacteroides* spp. are more prevalent in cows with uterine disease, but found that *E. coli* and *T. pyogenes* have very low prevalence (83) or are not identified (84). Nonetheless, when identified by PCR, both predispose to uterine disease (85,86). Therefore, it seems that, even in small numbers, both *E. coli* and *T. pyogenes* participate in the pathogenesis of uterine disease, likely because of synergistic effects with *F. necrophorum* and *Bacteroides* spp. (76,77,87–89). In fact, *E. coli* has been shown to increase the susceptibility of the endometrium to subsequent infection with *T. pyogenes* and *F. necrophorum* (57,68,83,85,90), and *T. pyogenes* acts synergistically with *F. necrophorum* and *Bacteroides* spp. to enhance the severity of uterine disease (76,77,87). In models of necrobacillosis, the lethal dose of *F. necrophorum* can be greatly decreased by co-infection with *E. coli*, *T. pyogenes*, or *Bacteroides* spp. (88,89). Among their effects, *E. coli* releases bacterial-wall lipopolysaccharides (LPS)(91), *T. pyogenes* produces the cholesterol-dependent cytotoxin pyolysin (PLO) (85,92), *F. necrophorum* produces a leukotoxin (LKT) (85), and *Bacteroides* spp. produce short-chain fatty acids that inhibit phagocytosis and killing of bacteria by neutrophils (93).

E. coli and *A. pyogenes* have been studied more extensively than the other bacteria. Recent work has highlighted the importance of *E. coli* in the development of metritis and endometritis in dairy cows (83,85,86,90,94), especially the fact that *E. coli* predisposes to infection with other pathogenic bacteria such as *F. necrophorum* and *T. pyogenes* (83,85,90), which increases the risk of developing metritis and endometritis. It was observed that *E. coli* that cause uterine disease are different from known entero-pathogenic *E. coli*. The utero-pathogenic *E. coli* are more adherent and invasive to endometrial cells than other *E. coli* and also stimulate greater production of PGE_2 and IL-8, an important neutrophil chemokine (94). Bicalho et al. identified 6 virulence factors present in *E. coli* to be associated with metritis and endometritis: fimbriae components H (fimH), hemolyn A (hlyA), cytolethal distending toxin (cdt), group II capsule (kpsMII), invasion of brain endothelium (ibeA), and arginine succinyltransferase (astA). Of all these virulence factors, fimH was the most significant because of the high prevalence in isolates from cows with metritis (87% of isolates) and the significant association with risk of uterine diseases, particularly when astA was also present. Cows with fimH-carrying *E. coli* had a 6.0-fold increase in the odds of having metritis compared with culture negative cows. If *E. coli* carried both fimH and astA, the odds of developing metritis increased 12.0-fold. The odds of developing endometritis were increased by 2.6- and 4.6-fold when *E. coli* carried fimH and astA, respectively (86). Later they observed that presence of fimH was associated with decreased reproductive performance (85).

T. pyogenes has been highlighted in several studies as the main causative agent of endometrial damage and infertility (77,82,87,95). In a recent study, Silva and co-workers tried to find specific virulence factors associated with uterine disease (96). They evaluated a series of virulence factors including PLO, neuraminidases (nan) nanP and nanH, collagen-binding protein A (cbpA), fimA, fimC, fimE, and fimG, but were unable to find any association with incidence of metritis. The group at Cornell University led by Dr Bicalho also tried to identify virulence factors present in *T. pyogenes*. They evaluated 5 known virulence factors and only fimA was found to be overrepresented in cows with metritis, while the other virulence factors were similarly found in both healthy and metritic cows (97). With the knowledge of the main uterine pathogens and their virulence factors, Dr Bicalho's group recently developed a vaccine that was shown to prevent metritis (98). They evaluated the efficacy of 5 vaccine formulations (3 administered subcutaneously and 2 intravaginally) containing different combinations of proteins (FimH present in *E. coli*; leukotoxin present in *F. necrophorum*, LKT; and pyolysin present in *T. pyogenes*, PLO) and/or inactivated whole cells (*E. coli*, *F. necrophorum*, and *T. pyogenes*) in preventing postpartum uterine diseases. They observed that all subcutaneous vaccines were able to reduce the incidence of puerperal metritis; however, intravaginal vaccination was ineffective (Figure 5). Reproductive performance was improved for cows that received subcutaneous vaccines as time to pregnancy was decreased in those cows (98). In general, vaccination induced a significant increase in serum IgG

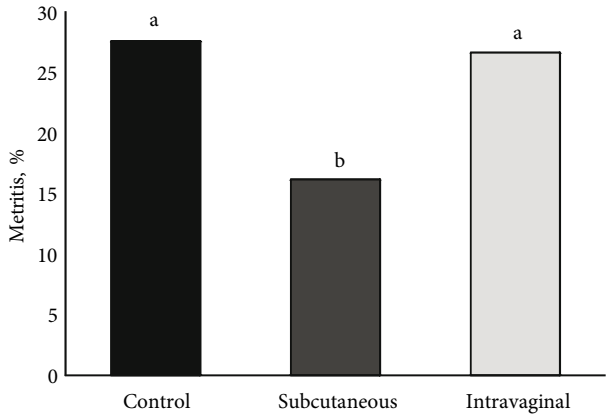

Figure 5. Metritis (fetid vaginal discharge and rectal temperature > 39.5 °C) incidence in the first 20 DIM in primiparous cows that receive 3 different subcutaneous vaccines (Vaccine 1, 2, and 3) or 2 intravaginal vaccines (Vaccine 4 and 5) at 230 and 260 days of gestation or that remained as untreated controls. Vaccine 1 was composed of inactivated bacterial whole cells (*E. coli*, *T. pyogenes*, and *F. necrophorum*) and proteins (FimH, PLO, and LKT); Vaccine 2 was composed only of proteins (FimH, PLO, and LKT); and Vaccine 3 was composed only of inactivated bacterial whole cells (*E. coli*, *T. pyogenes*, and *F. necrophorum*). Vaccine 4 was composed of inactivated bacterial whole cells (*E. coli*, *T. pyogenes*, and *F. necrophorum*) and proteins (FimH, PLO, and LKT), and Vaccine 5 was composed only of proteins (PLO and LKT). Different a,b letters above bars indicate significant difference; $P < 0.05$. Adapted from Machado et al. (98).

titers against all antigens, with subcutaneous vaccination again being more effective. In conclusion, subcutaneous vaccination with inactivated bacterial components and/or protein subunits of *E. coli*, *F. necrophorum*, *and T. pyogenes* can prevent puerperal metritis during the first lactation of dairy cows, leading to improved reproduction. The combined effect of bacterial infection and activation of inflammation is damage to the endometrium and embryo (99–101), delayed ovulation (67), shortened or extended luteal phase after ovulation (60,61,63,102), increased time to first insemination, decreased conception rates, increased time to conception (8,103–106), and increased pregnancy loss (106–108). Therefore, the best strategy to deal with the negative effects of uterine disease is to not have the disease in the first place.

7. Conclusion

In summary, the decrease in immune function may be associated with the decrease in glucose, glycogen, and calcium postpartum and the increases in NEFA and BHBA. Both endometrial cells and granulosal cells possess TLR that can recognize and mount an inflammatory response to PAMPs such as LPS or lipopeptides. Endometrial and leukocytic immune response is impaired early postpartum in cows that develop uterine disease. LPSs accumulate in the uterine lumen and can end up in the blood circulation and in follicular fluid. Exposure to LPS leads to endocrine dysregulation, which may affect steroidogenesis, ovulation, and luteolysis. There are specific *E. coli* that possess specific virulence factors such as fimH, hlyA, cdt, kpsMII, ibeA, and astA that cause uterine disease in dairy cows. There are specific *T. pyogenes* that possess specific virulence factors such as fimA in addition to PLO that cause uterine disease in dairy cows. *E. coli* and *T. pyogenes* act synergistically with *F. necrophorum* and *Bacteroides* spp. to cause uterine disease. A vaccine that contained inactivated *E. coli*, *T. pyogenes*, and *F. necrophorum* whole cells or their virulence factors fimH, PLO, and LKT was able to prevent metritis in dairy cows. The combined effect of bacterial infection and activation of inflammation is damage to the endometrium and embryo, delayed ovulation, shortened or extended luteal phase after ovulation, increased time to first insemination, decreased conception rates, increased time to conception, and increased pregnancy loss.

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