



Mini-Review Article

Endometrial Toll-like Receptors and Postpartum Uterine Infection in Bovine; a Minireview

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ABSTRACT

Postpartum uterine infection causes infertility and economic losses in the dairy industry. Since 2005, the host-pathogen interaction of uterine infection is investigated by understanding the innate uterine immunity through the interaction of endometrial Toll-like Receptors (TLRs) and the Pathogen associated molecular patterns (PAMPs) of the infectious agents. Transcriptional studies have confirmed the presence of most of the bovine endometrial TLRs suggesting that the uterus is equipped to mount TLR mediated response following infection. However, unlike cattle, buffalo lacks the expression of endometrial TLR1. Association between the phases of estrous cycle with the specific TLR gene suggests that ovarian steroids regulate the expression which is up-regulated following uterine infections. Of all the TLRs, the interaction between *Escherichia coli* lipopolysaccharide (LPS) and TLR4 is best studied till date and shows activation of pro-inflammatory cytokines, preferential production of PGE₂ and acute phase proteins in the bovine endometrium. Chronologically, intrauterine effects of LPS precede the discovery of TLRs. Emerging evidence indicates the presence of LPS in the follicular compartment in localized uterine infections; however, the vascular pathway carrying LPS of uterine origin to the ovary remains to be established. There is a scope for studying the other PAMPs of *E coli* with the cognate endometrial TLRs in the large ruminants. Similarly, no information on the interaction of PAMPs of recognized pathogens like *Arcanobacterium pyogenes* and *Fusobacterium necrophorum* with the corresponding endometrial TLRs is available. Expanding our understanding on the interaction of the common endometrial pathogens with the specific TLR in the endometrium is an obligatory pre-requisite for using specific TLR agonists in combating uterine infections.

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INTRODUCTION

The key determinants for excellent fertility in dairy cow herds is a healthy uterine environment, optimal estrus detection efficiency and ideal timing for breeding, since any afflictions of endometrium perturbs the normal reproductive functions leading to infertility or subfertility (Jabbour *et al.*, 2009; Sheldon *et al.*, 2009). The development of uterine disease is the equilibrium between the endometrial immune response and the virulence factors as well as the load of the causative microbe. Of the uterine diseases, postpartum (PP) metritis is by far, the most commonly encountered problem in the buffaloes (Raman and Bawa, 1977; Azawi, 2008). Apart from natural service, large scale implementation of artificial insemination coupled with unhygienic practice has resulted in an increased incidence of uterine infections in the buffaloes (Farca *et al.*, 1997) thereby adversely affecting the fertility by delay in uterine involution, prolonged PP estrus, reduced conception

rate and extended calving interval (Azawi, 2010). The occurrence and economic impact of clinical and subclinical endometritis (SE) in the PP cow has been the subject of many studies (LeBlanc *et al.*, 2002a; Kasimanickam *et al.*, 2004; Gilbert *et al.*, 2005; Hammon *et al.*, 2006; Williams *et al.*, 2007). Endometritis and associated inflammatory responses compromise the animal welfare, leading to infertility in the cows (Borsberry and Dobson, 1989; Sheldon and Dobson, 2004) and buffaloes (Usmani *et al.*, 2001; Azawi, 2010). In India, it has been predicted that about 18–40% of cattle and buffaloes were culled mainly due to infertility (Sharma *et al.*, 1993). The impact of abnormal calving on lactation yield and 305 days milk yield were hampered in buffaloes (Taraphder, 2002).

The innate immune response of the endometrium to pathogens involves an entire armory that includes complement system, antimicrobial peptides,

immunoglobulins, acute phase proteins and pattern recognition receptors (PRRs) (Wira and Fahey, 2004). Germ-line-encoded PRRs recognize conserved pathogen-associated molecular patterns (PAMPs) synthesized by microorganisms but not by the host (Beutler, 2009; Takeuchi and Akira, 2010). The detection of PAMPs by PRRs in the endometrial cells triggers the production of cytokines and chemokines, which orchestrate an immune response to clear infection. The PRRs currently comprise four main classes of soluble and membrane-bound molecules viz., Toll-like receptors (TLRs), (RIG-I)-like receptors (RLRs), Nod-like receptors (NLRs) and C-type lectin receptors (CLRs) of which the most studied are the TLRs.

Structural Biology of TLR

The TLRs belong to the subfamily Leucine-rich repeats (LRRs) that recognize conserved components of pathogens and are collectively called as pathogen associated molecular patterns (PAMPs) that initiate innate immune response. They belong to type I integral membrane glycoproteins and possess three major domains. The extracellular N-terminal domain consists of approximately 16–28 LRR and each LRR consists of 20–30 amino acids with the conserved motif (Kawai and Akira, 2010). Thus far, ten types of bovine TLRs have been identified and characterized (McGuire *et al.*, 2005; Vahanan *et al.*, 2008; Ganesan, 2013); each has its cognate ligands that activate specific intracellular signaling cascades resulting in upregulation of stimulus-specific cytokine(s) release to defend against invading pathogens.

(TLR2/TLR1 or TLR2/TLR5)[H2] forms heterodimer to recognize an array of PAMPs predominantly from both Gram positive bacteria which includes lipopeptides, peptidoglycan, glycolipids, lipoteichoic acid (Kovacs-simon *et al.*, 2011). The receptor dimerization is important, and tri-acetylated lipopeptides are usually bound by TLR2/TLR1, whereas di-acetylated lipopeptides are bound by TLR2/TLR6 (Jin *et al.*, 2007). Recently, it has been shown that endometrial TLR2 and 6 are significantly upregulated in the buffalo with uterine infections (Ganesan, 2013). However, it remains to be elucidated whether the upregulation of those TLRs is due to the PAMPs of Gram-positive *Archaeobacterium pyogenes*, the most common uterine pathogen.

Viral component of double stranded RNA (dsRNA) acts as a ligand for TLR3 and its synthetic analogue, polyinosinedeoxycytidylic acid I: C (Poly I: C) is widely used in *in vitro* studies. Uridine or guanosine-rich single-stranded RNAs from a wide range of viruses and synthetic imidazoquinoline-like molecules such as resiquimod are recognized by TLR7 and TLR8. Apart from LPS, use of other TLR agonists such as poly I: C and R848 in the treatment of endometritis has not yet been explored.

TLR Signaling Pathway

The LRR domains of TLRs are involved directly in the recognition of various components of microbes. The cognate ligands recognized by different TLRs are summarized in Table 1. Remarkably, despite the conservation among LRR domains, different TLRs can recognize several structurally unrelated ligands. The subcellular localization of different TLRs correlates to some extent with the molecular patterns of their ligands. TLR1, TLR2 and TLR4 are located on the cell surface and are recruited to phagosomes after activation

by their respective ligands. By contrast, TLR3, TLR7 and TLR9, which are involved in the recognition of nucleic-acid-like structures, are not expressed on the cell surface. For instance, TLR9 has recently been shown to be expressed in the endoplasmic reticulum, and is recruited to endosomal/lysosomal compartments after stimulation with CpG motif (Akira *et al.*, 2006).

Table: TLRs and their cognate ligands

TLR	Location	Type of ligands
TLR1	Plasma membrane	Triacylated lipopeptides
TLR2	Plasma membrane	Peptidoglycan, lipoteichoic acid
TLR3	Endosome	ss RNA and ds DNA
TLR4	Plasma membrane	LPS, LTA, fibronectin, mannan (Candida)
TLR5	Plasma membrane	Flagellin
TLR6	Plasma membrane	Diacetylated lipopeptides, fungal
TLR7	Endosome	ssRNA, imidazoquinolines
TLR8	Endosome	ss RNA
TLR9	Endosome	Bacterial and viral unmethylated CpG motif

Ligand recognition by TLRs leads to the recruitment of various TIR domain-containing adaptors such as myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor protein inducing IFN- β (TRIF) and TRIF-related adaptor molecule (TRAM). The engagement of TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR11 with their respective ligands recruits MyD88. In addition to MyD88, TLR1, TLR2, TLR4 and TLR6 recruit TIRAP, which serves as a linker adaptor between the TIR domain of TLRs and MyD88. Binding of ligand with TLR3 and TLR4 recruits TRIF. Furthermore, TLR4 recruits TRIF through TRAM, which links between TIR domain of TLR4 and TRIF. This recruitment of adaptors triggers the cascade of signaling pathway and ultimately the activation of transcription factors such as nuclear factor- κ B (NF κ B) and interferon regulatory factor (IRF) (Kawai and Akira, 2010; Takeuchi and Akira, 2010).

From clinical point of view, the cellular components of *E.coli* like LPS and flagellum will be recognized by TLR4 and 5, respectively before it colonizes the endometrium. The cellular component of *A.pyogenes* such as triacylated lipoproteins and peptidoglycans are the potential ligands recognized by TLR2/TLR1 or TLR2/TLR6 (Kovacs-Simon, 2011). TLR9 recognizes the unmethylated CpG motifs of single-stranded DNA present in the genomes of many viruses and bacteria probably of IBR/IPVV of Herpes virus family and *Brucella abortus* as they are rich in CpG motif. Hence, the role of TLR agonists in clearing the uterine infection deserves investigation.

Uterine Infection

Contamination of bovine uterus is a consistent feature during parturition. Apart from bacterial load, virulence factors (VF) are the important pathogen-based determinants of infection. Bacterial overload, which is common following dystocia and retained fetal membranes,

causes PP uterine infections (LeBlanc, 2008). Despite the fact that the culture of uterine samples yields a wide range of bacteria during the first 3 weeks PP, presence of *Arcanobacterium pyogenes*, *Escherichia coli*, *Fusobacterium necrophorum* and *Provetella spp* are commonly associated with clinical or subclinical endometritis (Griffin *et al.*, 1974; Bonnett *et al.*, 1991; Bicalho *et al.*, 2010). *E.coli* isolated from bovine uterus within day 10 PP expressed a battery of VF, of which, fim H gene has been shown to increase the risk of endometritis (Silva *et al.*, 2009; Bicalho *et al.*, 2010). Analogous to enteropathogenic and uropathogenic *E.coli*, an endometrial pathogenic *E. coli* (EnPEC) has been characterized recently and was found to be more adherent and invasive to endometrial cells and stimulated production of chemokines and PGE₂ in cell culture as well as mice model (Sheldon *et al.*, 2010). Bicalho *et al.* (2010) reported that six *E. coli* VFs were associated with uterine infection: fimH, astA, cdt, kpsMII, ibeA, and hlyA. The VF gene fimH was the most prevalent in cows with metritis. Apart from *E.coli*, *A. pyogenes* has been emphasized in several studies as the main causative agent of endometrial damage and infertility (Dohmen *et al.*, 2000). The VF that confers pathogenicity to *A. pyogenes* includes pyolysin (plo), a collagen-binding protein (CbpA), and a component of fimbriae (fimA). A hypothesis has been proposed that sequential infection of the PP uterus by the common bacteria determine the endometritis. For instance, *E. coli* infection during the first week of PP increases the susceptibility of the endometrium to subsequent infection with *A. pyogenes* (Olson *et al.*, 1984; Williams *et al.*, 2007), while *A. pyogenes* acts synergistically with *F.necrophorum* and *Provetella sp* to enhance the severity of uterine disease (Bonnett *et al.*, 1991). Among the effects, *E. coli* releases bacterial cell-wall components like LPS (Williams *et al.*, 2008); *A. pyogenes* produces the cholesterol-dependent cytotoxin, pyolysin (Bicalho *et al.*, 2011) and a growth factor for *F. necrophorum* (Sheldon and Dobson 2004); *F. necrophorum* produces a leukotoxin; and *Provetella sp* produces a substance that inhibits phagocytosis (Sheldon and Dobson, 2004). Thus, apart from host factors, bacterial load and/or VFs of the common endometrial pathogens determine whether an infection of the PP uterus would result in recognized inflammatory conditions like clinical or subclinical endometritis. Beside the above bacteria, bovine herpes virus IV (BoHV-4) has been linked to cause uterine disease based on cell culture studies (Donofrio *et al.*, 2007).

Endometrial TLRs

In cattle, the expression of TLR1 through 10 has been reported in the body of uterus, ipsilateral and contralateral horns (Davies *et al.*, 2008). In buffalo, the expression profile of TLR2, 5, 7, 8, 9 and 10 in the normal uterus has been reported without delineating the influence of estrous cycle and discriminating endometrium from the myometrium (Vahanan *et al.*, 2008). Barring Herath *et al.* (2006), others have studied the expression of TLRs only at transcriptional level. Immunohistochemical and western blot studies would throw much better information on the endometrial TLR expression. Further, *in vitro* and whole animal studies with agonist stimulation would indicate the functional competence of TLR proteins and unravel the immune

response following ligand binding. Expression pattern of various endometrial TLRs is markedly influenced by the sex steroids owing to the functional dependency of endometrium across the estrous cycle. Hence, ovariectomized animal will be an appropriate model to study the endometrial TLRs expression as the hormonal milieu can be modulated by exogenous administration of estrogen and/or P₄.

Transcripts of TLR4 and 5 showed highest expression during third trimester of pregnancy in cattle. Conversely, TLR 1/6 tend to show increased transcription during first trimester of gestation (Silva *et al.*, 2012) suggesting that the uterus may be more responsive to pathogens during the period of pregnancy and subsequent parturition (Davies *et al.*, 2008). An increased transcription of TLR 4 and 5 may be related to defense mechanism against such infections (King *et al.*, 2003). In the inter-cotyledonary region, variable levels of TLR 1/6, TLR3, TLR4, TLR5, TLR7, TLR8, and TLR10 transcripts were observed (Martins *et al.*, 2011). Oliveira *et al.* (2008) demonstrated that TLR9 has prominent role among the TLRs in attributing host resistance to *B.abortus* infection.

Most, if not all, the functional studies are confined to TLR4 as it recognizes the lipopolysaccharide component of *E.coli*. Administration of intravenous *E. coli* LPS disrupts neuroendocrine activity and results in interference with the estrous cycle. For instance, it is shown that LPS delayed ovulation, CL formation and rise in P₄. In addition, upregulation of caspase-3 transcripts is also shown to compromise CL function and luteolysis (Herzog *et al.*, 2012). Furthermore, CL diameter was smaller during the first PP cycle in cows with uterine pathogens suggesting an inimical effect of metritis on PP ovarian function (Williams *et al.*, 2007). In our opinion, intravenous administration of LPS, at best, mimics the septic shock induced by toxic puerperal metritis that occur within day 10 PP and cannot be equated with PP metritis or subclinical endometritis as they are localized infections with little systemic involvement.

Cows with *E. coli* infection after parturition had more LPS and PGE in peripheral circulation. The association between infection and PGE was supported *in vitro* since LPS treatment of endometrial explants and cells preferentially stimulated PGE rather than PGF concentrations (Herath *et al.*, 2009). It is well known that endometrial epithelial cells are the exquisite source of PGF as they harbor oxytocin receptors and stromal cells produce more PGE₂ (Fortier *et al.*, 2008). However, it is shown that endometrial epithelial cells accumulate more PGE than PGF in response to LPS. This switch in PG accumulation was associated with an increased level of phospholipase A2G6 (PLA2G6) protein in epithelial cells, rather than changes in the levels of PGES (PGE synthase) or PGFS (Herath *et al.*, 2009). It would be interesting to know how LPS, or any infectious stimuli, cause a switch from PGF to PGE in the epithelial cells.

It has been demonstrated that LPS in the follicular fluid decreases the aromatase expression in the granulosa cells, increases the secretion of PGE and PGF, and transcripts of pro-inflammatory cytokine resulting in altered ovarian function (Sheldon and Bromfield, 2011; Borges *et al.*, 2012; Jacca *et al.*, 2013). However, uterine infection does not affect peripheral plasma FSH concentrations or the consequent emergence of a wave of

growing follicles (Sheldon *et al.*, 2002; Williams *et al.*, 2007). The significance of localized uterine infection on the impairment of ovarian function is interesting as it confers LPS a 'hormone-like role'; hence, it is necessary to delineate how the LPS reach follicular compartment or ovary from an infected uterus. In other words, the relative importance of utero-ovarian pathway and general circulation has to be unraveled in relation to uterine infections. In addition to LPS, *E.coli* possesses various VFs like fimbriae, cell wall proteins and adhesins which are putative ligands for various TLRs. For instance, presence of fim H gene in the uterus on PP day 3 is associated with risk of metritis (Bicalho *et al.*, 2010). Hence, it is necessary to study the role of TLR5 vis-à-vis fimbriae (Davies *et al.*, 2008). Practically, no data is available on the PAMPs of *A. pyogenes*, *F necrophorum* or *Provetella sp* with the corresponding bovine endometrial TLRs. Presence of LPS in the follicular fluid and its adverse effect on follicular and CL functions ushers us to investigate the presence and role of other bacterial components in the ovarian compartment. Since myometrial contractions play an important role in eliminating the exudate, the effects of TLR-PAMP interaction on inducing contraction associated proteins in the myometrium deserves attention. It is worth mentioning that before the advent of TLRs, intensive investigation with intrauterine LPS has been done as a treatment for endometritis in the cattle (Dhaliwal *et al.*, 2001) and buffalo (Singh *et al.*, 2000).

Hormonal Regulation of TLR Expression

The uterus is exceptional among mucosal tissue on two accounts: first, ovarian steroid hormones have considerable effects on both afferent and efferent immune events (Wira and Rossoll, 1995). Thus, the outcome of an immune response and the elicitation of protective immunity to pathogens can be markedly influenced by the stage of the oestrous cycle at which priming or infection takes place. Second, uterus lacks highly organized secondary lymphoid nodules such as Peyer's patch of the ileum (Robertson, 2000). In the cattle, innate immune response of endometrium under luteal phase is reduced and secretory activity of the endometrial glands provide congenial milieu for the establishment of pathogens. On the other hand, the rising levels of E₂ in the follicular phase promote migration of immune cells into the uterine lumen, increasing uterine defenses (Liu *et al.*, 2012).

The transcripts of TLR2/TLR6, TLR3, 4, 5, 6, 7 and 9 were found to be higher in the endometrium during the secretory phase than that of luteal phase of the menstrual cycle implying that P₄ has a positive influence in the expression of TLRs (Nasu and Narahara, 2010).

CONCLUSION

Non-specific uterine infections during the postpartum period cause infertility and economic losses in the cattle and buffalo. The role of virulence factors in the establishment of infection by the common uterine microbes is emerging. Much of the expression studies on TLRs were done at mRNA level that needs to be corroborated at protein level by immunohistochemistry and Western blotting. Except LPS, other PAMPs of *E.coli*, *A pyogenes*, *F necrophorum* and *Provetella* have not been studied. Similarly, apart from TLR4, information on other TLRs is lacking. LPS released by the

infected uterus reaches ovarian compartment and adversely affects the endocrine functions of follicle and CL. Regulation of endometrial TLRs by ovarian steroids need to be investigated ideally in ovariectomized model. Potential of different TLR agonists in combating uterine infection needs to be explored in the bovine.

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