Toll-like receptors (TLR-2 and TLR-4): putative role in the uterine inflammatory response in cows – A mini review

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Abstract Metritis and endometritis are the most prevalent uterine inflammatory diseases in bovines and cause infertility and high economic loss to farmers. Many studies have been conducted to investigate the mechanism of pathology, immune response and therapeutics associated with these diseases. This review focuses mainly on the immune system present in the uterus and on the involvement of Toll-like receptors. This study aimed to provide a deep understanding of the structure, function and expression pathways of Toll-like receptors (TLR-2 and TLR-4) in the treatment of pathogenic microorganisms invading the uterus and their significance in inflammatory conditions such as metritis and clinical and subclinical endometritis. The genetic associations of TLRs with diseases and some therapeutic interventions are also briefly discussed. As such, TLRs-2 and 4 play a critical role in providing a uterine defense mechanism against foreign substances (embryos, sperm, and pathogenic bacteria). In the present era, the use of TLR agonists is being unfurled for treatment, and further exploration and study of this topic in the future are needed.

Keywords: reproductive health, infertility, immunology, inflammation

1. Introduction

The uterine environment is normally sterile (Sheldon et al 2008). Conditions such as parturition, insemination and mating facilitate the entry of foreign substances such as bacteria, viruses, fungi and sperm into the uterus (Sheldon et al 2020). Invading sperm, embryo formation and ovulation mediate inflammatory reactions in the female reproductive tract (Kannaki et al 2011). Endometritis, including clinical and subclinical endometritis, is the most common postpartum uterine disease (Piersanti et al 2019). Indeed, 80-90% of animals have bacteria in their uterine lumen during the first 2 weeks after calving (Sheldon et al 2018). Most of these bacteria are pathogenic and need to be cleared from the uterus. The body recognises these pathogens through the innate immune system present in the endometrium (Monie et al 2009, Sheldon et al 2014). Endometrial epithelial and stromal cells have pathogen recognition receptors (PRRs) called Toll-like receptors (TLRs) for the detection of microbes and thus produce an inflammatory response to bacteria (Herath et al, 2006). The microbes associated with uterine diseases include a range of gram-positive and gram-negative bacteria. Fusobacterium and Corynebacterium are highly prevalent pathogenic bacteria found in the uterus under inflammatory conditions (Pascottini et al 2020; Miranda-Casoluego et al 2019). The other predominant microbes are Escherichia coli, Arcanobacterium pyogenes, and Prevotella species, which act synergistically to cause uterine disease. Pathogen-associated molecular patterns (PAMPs) are specific markers of pathogens that are identified by pattern recognition receptors (PRRs) on host cells. Toll-like receptors are PRRs that work by forming dimers, heterodimers or homodimers. As a common uterine pathogen, E. coli has lipopolysaccharides recognised as PAMPs by TLR-4 expressed on the epithelial and stromal cells of the uterine endometrium (Herath et al 2006). Similarly, gram-positive (diacylated lipopolypeptides) and gram-negative bacteria (triacetylated lipopolypeptides) have lipopolypeptides as PAMPs, which are identified by TLR 2 (Nakayama et al 2012; Jin et al 2008). In endometrial cells, diacylated lipopolypeptides of gram-positive bacteria are detected through TLR 2/6, and triacylated lipopeptides of gram-negative bacteria are detected through TLR 2/1 (Turner et al 2014). It produces proinflammatory mediators such as cytokines and chemokines (Akira 2006), and this response is an important cause of endometritis in cattle (Turner et al 2014). This review aimed to understand the role of immune receptors (TLRs) in uterine inflammatory responses in cattle and what strategies we can adopt to minimise uterine diseases and improve fertility. This is significant because the
immunological pathways mediated by TLRs in the uterus play important roles in fertilisation, implantation and prevention of invading pathogens.

2. Uterine diseases in cattle

Metritis and endometritis are the most common postpartum uterine inflammatory diseases in cattle (Sheldon & Owens 2018). Uterine health in the postpartum period depends on the balance between immune defense and the pathogenicity of bacterial agents (Vallejo-Timaran et al 2021). Inflammation in response to pathogenic bacteria is a normal physiological process in the involution process of the postpartum uterus (Carneiro et al 2016). Endometritis is defined as uterine inflammation occurring >21 days after delivery and is diagnosed as clinical endometritis in the case of purulent vaginal discharge of uterine origin or subclinical endometritis if the cow does not exhibit vaginal discharge but has an increased proportion of polymorphonuclear cells that exceeds the operator-defined threshold and affects reproductive performance” (Williams 2013; Wagener et al 2017). “When cows have an abnormally enlarged uterus with foul-smelling and red-brown watery vaginal discharge within 21 days postpartum, it is called metritis” (Giuliodori et al 2013). According to the research of Vallejo-Timaran et al (2021), 51.2% of cows suffered from at least one postpartum uterine disease during the first 60 days after delivery. The incidence risks of metritis and clinical endometritis in this study were 25% and 29.4%, respectively (Vallejo-Timaran et al 2021). The prevalence of clinical endometritis varies from 9.6% (Dubuc 2010) to 28% (Ernstberger et al 2019) and 67.2% (Nyabinwa et al 2020). The prevalence of subclinical endometritis may reach 34% during the first 7 days after calving (Quintela et al 2017). Studies have shown culling incidence due to infertility at 200 days after delivery in 12.9% of cows (Vallejo-Timaran et al 2021) and at 300 days after delivery in 17.5% of cows (Dubuc et al 2011). Thus, endometritis and metritis result in low conception rates, a delay in the postpartum return of ovarian activity, high risks of culling and increased economic loss (Vallejo-Timaran et al 2021; Yáñez et al 2022).

3. Structure of TLRs in Cattle

Toll-like receptors belong to the family of leucine-rich repeat (LRR) proteins. Basically, Toll-like receptors (TLRs) encode type 1 transmembrane proteins of the interleukin-1 receptor family that consist of N-terminal leucine-rich repeats involved in ligand recognition, a transmembrane domain, and a C-terminal intracellular Toll/IL-1 receptor homologous domain for signal transduction (Fisher et al 2011; Gao et al 2017).
Toll-like receptors consist of three domains: an extracellular domain (ECD) that directly interacts with ligands, a Toll/interleukin-1 receptor (TIR) domain necessary for interaction with other TIR-containing systems and subsequent downstream signalling, and a transmembrane domain that anchors TLRs to the membrane (Federico et al 2020).

4. Types of TLRs in cattle

In cattle, TLRs 1-10 have been identified on endometrial cells, with TLRs 1-7 and 9 expressed on endometrial epithelial cells and TLRs 1-4, 6, 7, 9 and 10 expressed on stromal cells (Davies et al 2008). TLR2 recognises a wide variety of pathogens through peptidoglycans, lipopolysaccharides of gram-positive bacteria (Mogensen 2009). TLR 2 also triggers an inflammatory response to sperm entry into the uterus and during fertilisation (Shimada et al 2008). TLR5 recognises E. coli flagellin (Ajevar et al 2014). TLR3 recognises double-stranded RNA (Alexopoulos et al 2001). TLR7 and TLR8 recognise single-stranded RNA (Hemmi et al 2002; Heil et al 2003). TLR9 recognises unmethylated CpG-DNA motifs in the bacterial genome (Hemmi et al 2000; Takeda et al 2001; Wagner 2002). Bruce Butterler’s group identified TLR4 as the receptor for lipopolysaccharide (Poltorak et al 1998). TLR 2 and TLR 4 are the most important receptors found on the uterine endometrial cells of cows and are involved in various inflammatory responses in the uterus. TLR-4 and TLR-5 are highly upregulated during clinical and subclinical endometritis in buffalo in response to E. coli bacteria (Ajevar et al 2014).

5. Function and expression of Toll-like receptors

TLRs provide innate immunity to the genital tract, as they can recognise pathogen-associated molecular patterns (PAMPs). The binding of TLRs to PAMPs leads to a cascade of signal transduction for mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NFkB) transcription factors, leading to the secretion of prostaglandins, cytokines, chemokines and B and T-cell activation (Federico et al 2020; Komurcu et al 2016). However, there are other mediator proteins that help in the binding of PAMPs to TLRs, such as CD14 and MD2, in the case of lipopolysaccharides (LPS) ligands (Kim et al 2007). Proinflammatory mediators such as cytokines and chemokines direct the immune response to prevent the propagation of pathogens and eliminate them from tissues (Akira et al 2006; Beutler et al 2004). In bovines, when TLR4 binds to LPS and TLR1, TLR2 and TLR6 bind to lipopeptides, leading to the secretion of IL-8, IL-6 and PGE2 (Herath et al 2006; Cronin et al 2012). Chemokines such as IL-8 attract neutrophils and macrophages to the infected site or damaged tissue. IL6 has multiple functions, such as activating neutrophils and stimulating the acute phase response (Beutler 2009). However, danger signals and endogenous damage-associated molecular patterns (DAMPs), such as high-mobility group box 1 protein (HMGB1), ATP, nucleic acids and IL1α, which are released from damaged or dead cells, are also thought to bind pattern recognition receptors to initiate an inflammatory response (Chen & Nunez 2010). The most important TLRs for the recognition of bacteria are TLR1, TLR2 and TLR6, which form heterodimers to bind bacterial lipopeptides, and TLR4, which binds the cell wall component of the gram-negative bacterium lipopolysaccharide (LPS, endotoxin) in complex with CD14 and MD2 (Takeuchi & Akira 2010).

6. Putative role in uterine inflammatory diseases

The immune system is present in the female reproductive tract and works against pathogens, allogenic sperm (Elweza et al 2018) and fetuses (Ochiel et al 2008). The female reproductive tract is well equipped with both innate and adaptive immune responses. Innate immunity in the uterus plays an important role in protecting females from infections. Innate immunity is mediated by signalling molecules such as prostaglandins, cytokines, and phagocytic cells such as polymorphonuclear neutrophils and macrophages (Akthar et al 2021). Bacterial contamination of the uterine lumen is common in cattle following parturition and depends on the balance between the innate immune response and the regulation of inflammation (Lima 2020; Sheldon et al 2020). Some females produce an appropriate immune response and are able to clear pathogens, while other females develop chronic inflammatory conditions that lead to compromised fertility (Gilbert et al 1998; Sheldon et al 2009). Metritis and clinical and subclinical endometritis are common uterine inflammatory diseases found in cattle. E. coli and Arcanobacterium pyogenes are common bacteria that cause endometritis (Sheldon et al 2009). The ligands (PAMPs) associated with these bacteria are identified by TLRs that produce an immune response that subsequently regulates the activation of both innate and adaptive immunity (Vasselon et al 2002; Takeda et al 2003; Kaisho et al 2006). The initial defense of the endometrium against invading microbes is dependent on the innate immune system and includes recognition by host TLR proteins and secretion of antimicrobial peptides, cytokines, and acute phase proteins (Davies et al 2008, Sheldon et al 2009), which are important for the recruitment of other immune cells (Takeda et al 2003). In addition, TLR4 mRNA expression is upregulated in endometritis patients, which indicates that TLR4 activates bacteria. This activation of TLR4 leads to a switch in PGF2α to PGE2, which may be one of the reasons for the long luteal phase in endometritis (Davies et al 2008; Herath et al 2009; Kannaki et al 2011).

PGF2α is a luteolytic hormone, while PGE2 is a luteotrophic hormone that causes persistence of the corpus luteum, resulting in a long luteal phase and anestrus. Additionally, progesterone is maintained in the luteal phase, which further decreases immunity, thus facilitating the proliferation of pathogens (Adnane et al 2022).
Moreover, uterine infection affects the secretion of reproductive hormones. TLR-4 on granulosa cells has specific receptors for LPS, thus triggering an inflammatory response in follicular cells, which compromises the steroidogenic activity of the ovary (Bromfield et al 2015). Severe uterine contamination is associated with a decreased number of follicles and low levels of estrogen and progesterone, resulting in infertility and subfertility (Williams et al 2007).

TLR-4 present in endometrial epithelial and stromal cells acts as a receptor for lipopolysaccharides produced by *E. coli* bacteria, which are the most common pathogens involved in uterine diseases. TLR-4 binds to lipopolysaccharides, which causes the release of proinflammatory cytokines and chemokines. Chemokines stimulate neutrophils and macrophages to eliminate bacteria from the uterus (Umer et al 2022), and inflammatory reactions lead to endometritis and metritis. TLR-2 recognises cell surface ligands of gram-positive bacteria and triggers an inflammatory response (Akira et al 2006). Sperm entry into the uterus also triggers an inflammatory response (Akhtar et al 2020; Eleshe et al 2021). In the uterus, TLR-2 is detected in the endometrial glands. This approach is very important for removing ‘less fit’ sperm and preventing polyspermy to some extent (Akhtar et al 2020).

7. Relation of TLRs to the genetic makeup of animals

Many studies have been performed on the relationship between uterine inflammatory diseases and the genetic makeup of animals (Pinedo et al 2013). This relationship can be very helpful in the genetic selection of animals for uterine disease resistance. Given that fertility is a low heritable trait and cannot be determined based on phenotype, specific biomarkers are needed to genetically select animals with good fertility and disease resistance (Pohler et al 2020). Known variation within seven bovine TLR genes recognising pathogen-associated molecular patterns does not modulate large effects on risk for uterine disease. Weak associations were observed between SNPs (single-nucleotide polymorphisms) occurring in four bovine innate immune genes (TLRs 2, 4, 6, and 9) and uterine health in Holstein dairy cows, suggesting that variation in bovine innate immune genes may modulate small effects on the incidence of uterine disease (Pinedo et al 2013). Another study showed that there was no difference in the expression of the innate immune response agent between the fertile and infertile groups, except for TLR4, which is required for the detection of LPS (Herath et al 2006). However, further studies need to be performed in this field to come to a clearer conclusion.

8. Future opportunities for therapeutic interventions

In the present era, the global threat of antimicrobial resistance dissemination follows the World Health Organisation guidelines on the use of medically important antibiotics (WHO 2017). Therefore, alternative therapies for uterine diseases need to be developed to reduce the use of antimicrobial agents in dairy cows. The role and mechanism of TLRs in uterine inflammatory diseases provide opportunities for targeted therapeutic interventions. To date, TLRs have been applied in many inflammatory diseases and autoimmune diseases and as vaccine adjuvants (Kanzer et al 2007). Modulating TLR signalling pathways could help enhance the innate immune response and promote more effective clearance of pathogens.

One approach is the development of TLR agonists, which are synthetic molecules that mimic the action of specific TLR ligands. These agonists can potentially stimulate TLRs and enhance the immune response against uterine pathogens. TLR4 agonists include synthetic analogs of Lipid A, such as MPLA and AGPs (Federico et al 2020). Conversely, TLR antagonists can be explored to dampen an overactive immune response, prevent tissue damage and promote healing. Some TLR4 antagonists are Eritoran and TAK-242 (Okada et al 2019). TLR4 antagonists, such as adenosine and the codycepin parthenolide, have anti-inflammatory effects (Abdul-Careem et al 2011; Fan et al 2017). MAPK inhibitors are promising therapeutic agents for inflammatory diseases in which bacterial products and proinflammatory cytokines play critical roles in pathogenesis (Oladejo et al 2021). The therapeutic invention of NF-κB inhibitors for treating inflammation and cancer has been implemented in several clinical trials (Oladejo et al 2021). The antagonistic mechanism of RsLPS, a natural, nontoxic molecule of LPS produced by *Rhodobacter sphaeroides* (RsLPS), is thought to involve competition with toxic LPS for the same binding site on the MD2 receptor. The targets for competition may include CD14, TLR4 or other receptor components (Lohmann et al 2003). Treatment of cows with postpartum uterine infection with 150 μg of LPS improved their reproductive status (Moraes et al 2017). Therefore, the use of LPS analogs or LPS antagonists to inhibit the LPS-receptor complex has been widely studied for the development of novel therapeutic drugs (Rallabhandi et al 2012; Lu et al 2013; Anwar et al 2015). However, further research is needed to uncover the mechanisms underlying TLR-mediated immune responses in uterine infections. Understanding the intricate interplay between TLRs, other immune cells, and the local uterine environment could lead to the development of novel therapeutic strategies that specifically target uterine infections in cattle.

9. Conclusions

Endometritis and metritis are the most prevalent disease conditions in cattle during the postpartum period. These conditions can lead to infertility and subfertility and hence major economic losses. Toll-like receptors (TLRs) are involved in pathogen recognition in the uterus and are mediators of the first line of defense. These receptors induce an inflammatory response against invading pathogens, ultimately paving the way for further defence mechanisms to operate to neutralise
pathogens. Unearthing more knowledge in the area of polymorphisms of the TLR gene in cattle and their relation to uterine disease conditions will be highly helpful in the genetic selection of disease-resistant cattle breeds. Further studies on the modulation of TLR signalling to better understand and improve the future use of TLR agonists or antagonists could lead to improved alternative treatment strategies for endometritis in cattle.

Ethical Considerations

Not applicable.

Conflict of Interest

The authors have no conflicts of interest to declare.

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