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Running title: Toll-like receptors in the bovine corpus luteum

Toll-like receptor and related cytokine mRNA expression in bovine corpora lutea during the estrous cycle and pregnancy

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Abstract

Improving our understanding of the mechanisms controlling the corpus luteum (CL) and its role in regulating the reproductive cycle should lead to improvements in the sustainability of today's global animal industry. The corpus luteum (CL) is a transient endocrine organ composed of a heterogeneous mixture steroidogenic, endothelial and immune cells, and it is becoming clear that immune mechanisms play a key role in CL regulation especially in luteolysis. Toll-like receptors (TLR) mediate innate immune mechanisms via the production of pro-inflammatory cytokines, especially within various tissues, although the role of TLR within CL remains unknown. Thus, the objectives of this study were to characterize TLR mRNA expression in the CL during the estrous cycle and in pregnancy day 30-50, and to examine the role of TLR signaling in luteal cells. Corpora lutea were collected at various stages of the cycle and pregnancy and analyzed for TLR and cytokine mRNA expression. In addition, luteal cells were cultured with the TLR4 ligand (lipopolysaccharide, LPS) for 24 h to evaluate the role of TLR4 in regulating luteal function. Toll-like receptors 1, 2, 4, 6, tumor necrosis factor alpha (TNF), interferon gamma (IFNG), and interleukin (IL)12, mRNA expression was greatest in regressing CL compared with earlier stages ($P<0.05$), whereas no change was observed for IL6 mRNA expression. Cytokine mRNA expression in cultured luteal cells was not altered by LPS. Based on these data, one or more of the TLRs found within the CL may play a role in luteolysis, perhaps via pro-inflammatory cytokine mRNA expression.

Keywords: Toll-like Receptors, Corpus Luteum, Cytokines, Luteal Immune Response

Introduction:

Understanding the complex molecular regulatory mechanisms involved in the maintenance or regression (luteolysis) of the bovine corpus luteum (CL) is essential to the development of improved methods for enhancing reproductive efficiency of dairy cattle, especially since continued secretion progesterone by the CL is critical for embryonic survival and the successful outcome of pregnancy. Luteolysis has been described as a pro-inflammatory event (Walusimbi and Pate, 2013), and there is strong evidence to show that the pro-inflammatory cytokines (e.g. Tumor Necrosis Factor (TNF) Interferon gamma (IFNG) and Interleukin 1 beta (IL1B)) display elevated levels of mRNA expression in bovine CL undergoing natural or prostaglandin (PG) F-2α-induced luteolysis (Petroff et al. 2001; Neuvians et al., 2004). Furthermore TNF and IFNG treatment of bovine luteal cells in vitro inhibited progesterone production in a dose dependent manner, indicating that these cytokines have luteolytic actions (Petroff et al., 2001; Skarzynski et al., 2008; Walusimbi and Pate, 2013). Lastly, TNF and IFNG have been shown to act synergistically to promote luteal cell death (Petroff et al., 2001; Walusimbi and Pate, 2013). One ligand/receptor system that regulates inflammation in tissues are the Toll-like Receptors (TLR) and their ligands. In view of their involvement in promoting inflammation and our need to investigate further the control of inflammation during luteolysis in the bovine CL, we chose to examine the mRNA expression of these receptors in bovine CL throughout the estrous cycle and during pregnancy.

Toll-like receptors are critical components of the innate-immune system, allowing host cells to recognize and mount an appropriate response (e.g. inflammation) to microbial pathogens, that the host organism may encounter (Aflatoonian and Fazeli 2008; Kawai and Akira 2010). Toll-like receptors are members of the pattern recognition receptor (PRR) family that have evolved to recognize protein domains/sequences on various pathogens, which are known as pathogen-associated molecular patterns (PAMPs) (Aflatoonian and Fazeli 2008; Kawai and Akira 2010). There are currently 10 (human, bovine) or 12 (mouse) different TLR, each of which has a relatively well-defined function (Aflatoonian and Fazeli 2008; Davies et al. 2008; Kawai and Akira 2010). Toll-like receptors 1, 2, 4 and 6 are associated with the plasma membrane and enable recognition of a variety of PAMPS produced by bacteria, viruses, fungi or parasites. Additionally, the lipopolysaccharide (LPS) and triacyl lipopeptides produced by gram negative bacteria such as E. coli is recognized by TLR4, and by TLR1 and TLR2 heterodimers, respectfully, and diacyl lipopeptides of gram positive bacteria are recognized by TLR2 and TLR6 heterodimers (Aflatoonian and Fazeli 2008; Kawai and Akira 2010). On the other hand, TLR3, 7 and 8 are intracellular receptors and recognize internalized bacterial or viral nucleic acids (Aflatoonian and Fazeli 2008; Kawai and Akira 2010). Little is known about the function of, or ligand for, TLR 10, but it is homologous to TLR2 and is likely a TLR2-associated receptor (Aflatoonian and Fazeli, 2008). However, TLR (e.g TLR2 and 4) activation has also been shown to occur in response to the products of normal cellular degradation, such as heat shock proteins (HSP) and high mobility group box 1 (HMGB1) proteins, and thus TLR-mediated pro-inflammatory cytokine production may occur quite independently of microbial infection (Kawai and Akira, 2010). Furthermore, since luteolysis involves widespread cellular degeneration, it is likely that the regressing CL during the estrous cycle would represent a rich source of potential endogenous ligands to activate TLRs expressed within this tissue in otherwise healthy animals.

The responses of Toll-like receptors to microbial pathogens have been studied in reproductive tissues such as the bovine uterus (endometrium) and ovary (Battaglia et al. 2000; Sheldon et al. 2002; Herath et al. 2007; Davies et al, 2008; Sheldon et al., 2009). These investigators demonstrated several TLRs in the endometrium and examined their role in response to pathogens in cattle with uterine infections (e.g. endometritis, metritis or pyometra). Lipopolysaccharide (LPS), produced by E. coli, a common causative agent of uterine infections in dairy cows, binds to TLR4 on uterine epithelial cells. This induces a pro-inflammatory cascade of cytokines leading to altered prostaglandin (PG) production (increased PGE-2:PGF-2α ratio) by the uterus (Davies et al, 2008; Sheldon et al., 2009) which causes delayed luteolysis and extended cycles in animals with uterine infections (Davies et al, 2008; Sheldon et al., 2009; Saut et al., 2014). In other studies it was shown that LPS concentrations in peripheral blood (Mateus et al. 2003), and within the ovarian follicular fluid (Herath et al. 2007), are elevated in animals with uterine infections, suggesting that LPS ***may*** also have <u>***direct***</u> effects on ovarian function. Indeed, estrogen production and follicular growth is reduced in animals with uterine infections or in response to LPS infusion (Battaglia et al. 2000; Sheldon et al. 2002; Herath et al. 2007). It is also known that smaller follicles produce smaller CL with lower serum progesterone concentrations (Perry et al. 2005; Robinson et al. 2005), which may help to explain lower than normal luteal phase progesterone concentrations observed in animals with uterine infections (Sheldon et al. 2009). Furthermore, LPS has been shown to exert direct actions on the CL as shown by studies in which bovine luteal cells were cultured with increasing doses of LPS, resulting in increased progesterone secretion (Grant et al. 2007). Taken together, these data suggest that in cows with uterine infections, microbial pathogens such as LPS, may also exert direct effects on the CL.

In view of the importance of improving our understanding about the role of cytokines and their role in the inflammatory luteolytic process, the overall goal of the current study was to examine the potential roles that TLR may have within the CL during the bovine estrous cycle versus pregnancy, with particular emphasis on their possible roles, via pro-inflammatory actions, in the control of luteolysis. This study was designed to carry out the following ***Primary Objectives***:

1) To determine the steady state levels of mRNA expression of multiple TLR and cytokines in the bovine CL collected at different stages of the estrous cycle and pregnancy, and

2) To examine the effects of LPS on cytokine mRNA expression by bovine luteal cells in culture.

Materials and Methods:

Tissue Collection

Ovaries were collected from a local abattoir (Martin's Abattoir and Wholesale Meats, Godwin, NC). Approval to obtain specimens from this official establishment was granted by the North Carolina Department of Agriculture and Consumer Services Meat and Poultry Inspection Division. Corpora lutea (CL; n=51) were collected and catalogued by stage (I, II, III, IV, or Pregnant (P)) based on appearance, ovarian and reproductive tract morphology (Ireland et al. 1980). Corpora lutea collected at stage I (~days 1-4:

n=7), stage II (~days 5-10: n=11), stage III (~days 11-17: n=11), stage IV (~days 18-20: n=13), and from pregnancy (~ 30-50 days: n=7), were used for these experiments. Corpora lutea of pregnancy were confirmed by the presence of embryos in the uterus. Stage of pregnancy was estimated by conceptus size or crown-rump length measurement (by Dr. Peter Farin). Following collection, luteal tissues were frozen at -80˚C until analysis for mRNA or progesterone concentrations. Additional mid-stage (stage II; n=6) CL were collected and placed into ice-cold Hams F-12 media (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) during transport to the laboratory to be dissociated for culture experiments.

Dissociation of corpora lutea

Corpora lutea were collected and dissociation of luteal tissue was performed according to Pate (1993). Luteal tissue was minced and placed in 24 mM HEPES-buffered Ham's F-12 culture medium (Gibco, Invitrogen) containing 0.5% BSA (Sigma–Aldrich, St. Louis, MO, USA), 20 μg/ml gentamicin (Gentamicin Reagent Solution; Invitrogen), and 2000 U/g tissue collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ, USA). Pate (1993) demonstrated that this method of luteal cell isolation results in highly enriched populations of small and large luteal cells. Smaller cell types, endothelial cells, fibroblasts, immune cells, are removed during the slower speed centrifugation process (Pate 1993; Poole and Pate, 2012). Enrichment of small and large luteal cells as previously described (Pate, 1993; Poole and Pate, 2012) was confirmed in this set of experiments. Following dissociation, luteal cells were re-suspended in Ham's F-12 culture medium and cell viability was determined via standard viability stain (trypan blue; Sigma–Aldrich). Cells were placed in a 0.5% trypan blue solution, and counted on a hemacytometer according to Pate (1993) with cell viability routinely ≥ 80% live cells.

Luteal cell culture

Luteal cells (1.0x10^6 cells/ml) were plated to approximately 75% confluency in 24 well plates in 24 mM HEPES-buffered Ham's F-12 culture medium (Gibco, Invitrogen) containing 5% FCS (Sigma–Aldrich), 20 μg/ml gentamicin (Gentamicin Reagent Solution; Invitrogen) and incubated for 24 h at 37°C and 5% CO_2 in air. After 24 hr, media was replaced to remove dead cells and debris. Luteal cells were treated with LPS (TLR ligand tested; cat # L3024; Sigma–Aldrich) at 0, 0.01, 0.1, 1 μg/ml concentrations and were incubated at 37°C and 5% CO_2 in air, for additional 24 hours. These LPS doses have been shown to be effective in increasing progesterone secretion by bovine luteal cells in culture (Grant et al. 2007). After culture, media were removed and luteal cells were harvested to quantify cytokine mRNA by quantitative PCR (qPCR). Culture experiments were repeated a total of three times using CL from different animals.

Total RNA extraction and PCR

A. RNA Extraction and semi-quantitative PCR - bovine CL whole tissue samples

RNA was extracted from luteal tissue (approximately 100 mg) using 1ml of TRIzol (Sigma–Aldrich) and homogenization as described by Crosier et al., (2002). Following RNA isolation, RNA pellets were allowed to dry at room temperature for 5 minutes and were then dissolved in nuclease free diH_2O (Sigma–Aldrich) in volumes ranging from 10-60 μl based on pellet size. The concentration of RNA, and the 260nm : 280nm OD ratio, were determined by Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA samples were then subjected to DNase treatment followed by cDNA synthesis and semi-quantitative PCR as described previously (Crosier et al. 2002). For optimum synthesis efficiency, 1 μg of RNA was reversed transcribed in a total volume of 20 μl per reaction in accordance with manufacturers' recommendations. RNA was determined to be of high quality based on a 260:280 ratio of 1.8 or more, and this was confirmed by the presence of clear 28S and 18S rRNA bands on denaturing agarose gels stained with ethidium bromide. Primers for this procedure were synthesized based upon the published GenBank sequences to produce the expected product sizes (see Table 1). All primers were validated for semi-quantitative (sq) RT-PCR by running each primer at 20, 25, 30, 35 and 40 cycles and choosing a cycle number that corresponded to the linear range of amplification (36 cycles for all primer sets, except for the housekeeping gene, Beta Actin (ACTB) which was 33 cycles). Once primers were validated, sq RT-PCR was carried out using 2 μl of the cDNA product and the Taq PCR Mastermix® Kit (Qiagen Sciences, Valencia, CA, USA). Thermocycler conditions were as follows: 3 minute initial denaturation 94°C, followed by 33 or 36 cycles: denaturation, 30 seconds 94°C; annealing, 1 minute 50°C; extension, 1 minute 72°C; followed by a 5 minute 72°C final extension. Amplicons were separated on 2% agarose gels and stained with ethidium bromide. Signal intensities were quantified using Lab-Works imaging system (UVP Imagining Company, Upland, CA, USA). Values were calculated as the ratio of amplicon band intensity/ACTB band intensity; ACTB was used as a housekeeping gene since its levels of mRNA expression did not vary with stage of estrous cycle.

B. RNA Extraction and quantitative (Q)-PCR - bovine CL whole tissue and cell culture samples

Total RNA was extracted and purified from luteal tissues at various stages of the estrous cycle/pregnancy and the cultured luteal cells, using the RNeasy® mini kit (Qiagen Sciences, Valencia, CA, USA) according to manufacturer's recommendations. DNase treatment (Turbo DNA-free kit, Life Technologies, Carlsbad, CA, USA) was performed and RNA concentrations and purity determined using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Random-primed, reverse-transcribed (RT) cDNA synthesis in 20 μl reactions were performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's recommendations. For optimum synthesis efficiency, 1 μg of RNA was reversed transcribed in a total volume of 20 μl per reaction in accordance with manufacturers' recommendations. Forward and reverse primers directed toward Bos taurus TLR 1, 2, 4 and 6, TGFB, IFNG, TNF, IL6 and IL12A (Table 2; Integrated DNA Technologies, Coralville, IA, USA) were designed specifically for Q-PCR. Final concentrations (300 nM) of the forward and reverse primers were determined for each primer pair based upon optimal amplification efficiency (> 95%). Following the RT reaction, Q-PCR was performed on the LightCycler 480 II (Roche Diagnostics, Indianapolis, IN, USA) using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with 2 μl of the cDNA product in a 20 μl reaction volume under the following conditions: 95 ^{o}C for 5 min, 40 cycles of 94 ^{o}C for 30 s, 57 ^{o}C for 45 s, and 72 ^{o}C for 60 s, followed by an extra elongation of 5 min at 72 ^{o}C. Annealing temperature was optimized for each primer set. Primers were optimized using a temperature gradient from 51^{o} to 61˚C and checked for specificity based on the appearance of a single band via 1.2% agarose gel in 1X TAE buffer. Optimal annealing temperature for all primers was 57°C with a 300 nM primer concentration. Melting curves were generated for each run. The Q-PCR amplification products were electrophoretically separated on 1.5% agarose gels and visualized with ethidium bromide under UV light to ensure a specific band corresponded to the size of the expected cDNA fragment. Specific bands were extracted and purified using QIAquick gel extraction kit (Qiagen Sciences, Valencia, CA, USA). Following extraction, Q-PCR products were sequenced to confirm product specificity. A control sample that was not reverse transcribed was used to confirm that the product obtained was not amplified from genomic DNA. The Ct (the cycle number at which the fluorescence exceeds a threshold level) was determined for each reaction (run in triplicate) using the LightCycler 480 software and quantification was accomplished by normalization of Q-PCR data for each TLR and cytokine to that of ribosomal protein L19 (*RPL19)* RNA (housekeeping gene), using the $\Delta\Delta$Ct method (Livak and Schmittgen 2001). Ribosomal protein L19 was shown to be a constitutively expressed gene across all CL from different stages of the cycle and pregnancy. Q-PCR data from the luteal cell cultures studies were expressed as fold-difference compared with the control treatment (0 μg/ml of LPS).

Progesterone Radioimmunoassay

The method for the extraction and assay of CL progesterone concentrations was adapted from previously published methods (Estill et al. 1995; Garverick et al., 1985). Briefly, approximately 100 mg of luteal tissue was immersed in 10ml of cold 100% ethanol and homogenized. After centrifugation, the ethanol extract was dried under nitrogen and re-constituted in radioimmunoassay buffer (phosphate buffered saline plus 0.1% gelatin; PBS-gel; Estill et al, 1995). CL samples and progesterone standards (25-

2000 pg/ml) were assayed in duplicate using the Coat-a-count RIA kit (Coat-a-count, Diagnostic Products Corporation, Los Angeles, CA, USA) following the manufacturers instructions (Richards et al., 1994; Estill et al., 1995). All samples were run in a single assay and the intra-assay coefficient of variation was 6.3%. The sensitivity of this assay was 10 pg progesterone per tube (Richards et al., 1994). This assay has also been validated in our laboratory for use with bovine serum in a single assay (Whisnant and Burns, 2002; Lyons et al., 2016). Data are reported as nanograms of progesterone/mg luteal tissue.

Statistical Analysis:

All statistical analyses were performed using the mixed model of SAS (Statistical Analysis System Institute, Cary, NC, USA). mRNA expression data were log-transformed and analyzed using covariate analysis, with RPL19 as the covariate for Q-PCR and ACTB as the covariate for semi-quantitative PCR. One-way ANOVA was performed to determine differences among stages of luteal development for progesterone concentrations. In addition, a correlation analysis (PROC CORR) was conducted to determine the relationship between the mRNA expression of TLR and pro-inflammatory cytokines within bovine luteal tissue. Cell culture experiments were independently replicated with cells from different animals a minimum of three times unless otherwise stated. Additionally, the cell culture experiment was analyzed for linear, quadratic, and cubic relationships using a PROC GLM with orthogonal contrast for respective relationships. Differences were considered significant at $p < 0.05$, and a statistical tendency was declared when $0.05<p<0.1$.

Results:

Progesterone Concentrations within bovine CL:

Whole tissue CL progesterone concentrations were significantly decreased to 6.5 $\pm$ 2.2 ng/mg in stage IV CL, from ~ 30-40 ng/mg observed in CL from stages I, II, III, and in pregnancy ($p<0.001$). These CL progesterone concentrations are in agreement with those published by Garverick and colleagues (Garverick et al., 1985) in the bovine CL, who used a similar CL extraction procedure for progesterone.

TLR 1, 2, 4 and 6 mRNA expression:

The steady state mRNA expression levels of Toll-like receptors 1, 2, 4 and 6 mRNAs were examined in whole tissue CL samples via Q-PCR (Fig. 1). Toll-like receptor 1 mRNA expression was significantly increased in stage IV compared to stages I, II and III ($p<0.01$). Similar stage dependent profiles were seen for *TLR2*, *TLR4* and *TLR6* with a significantly increased at stage IV compared to stages I, II, III, and pregnancy ($p<0.01$; $p<0.05$; $p<0.05$; respectfully). There was also a tendency for pregnancy to show increased *TLR6* mRNA expression over stage II ($p< 0.1$).

TLR3, 7, 8 and 10, mRNA expression:

Analysis of steady state mRNA levels of *TLR3, 7, 8* and *10* was carried out by semi-quantitative (sQ) PCR, and the data are summarized in Table 3. No significant differences were observed between the stages of the cycle or pregnancy for any of these TLRs. *TLR3* mRNA expression at stage IV tended to be lower ($p< 0.1$) than stage III. *TLR8* mRNA expression tended to be lower in pregnancy compared to stage IV ($p< 0.1$).

MD2, CD14 and CD45 mRNA expression:

An examination of the expression levels of *MD2, CD14 and CD45* mRNAs was also carried out by semi-quantitative (sQ) PCR (Table 3). Lymphocyte antigen (*MD2)* and *CD14* were examined since they represent critical components of *TLR4* signaling. Expression of *MD2* was signficantly increased at stage III compared to stages I, II, IV and pregnancy ($p<0.05$). *CD14* showed a similar increase (significant) at stage III compared to stage IV ($p<0.01$). Cluster of Differentiation (CD) *45* (non-specific marker of leukocytes) mRNA expression was significantly increased ($p=0.01$) from stage I to stage III and there was a tendency ($p<0.1$) for stage III to be increased compared to stage II. Expression of *CD45* at Stage IV was significantly decreased compared to stages II, III, and pregnancy ($p<0.05$).

<u>*Cytokine mRNA expression:*</u>

Interferon gamma, *IL6*, *IL12* and *TNF* mRNA expression was also examined in whole tissue CL samples by Q-PCR (Fig. 2). IFNG was significantly increased at stage IV compared to stages I, II, III, and pregnancy ($p<0.05$). Interleukin *12* mRNA expression displayed a similar profile with a significant increase observed at stage IV vs. stages I, II, III, and pregnancy ($p<0.01$). Tumor necrosis factor mRNA expression also showed a significant increase at stage IV compared with stages I, II, III, and pregnancy ($p<0.01$). The cytokine *IL6* tended to be decreased in pregnancy compared to stage I ($p<0.1$) but showed no other stage dependent changes. There was no stage-dependent differential expression seen for TGFB mRNA expression (Fig. 2). An analysis of correlation between TLRs and proinflammatory cytokines (IFNG, IL6, 12, and TNF) mRNA expression in bovine CL tissue revealed a significant positive correlation ($p < 0.001$) between TLR1, 2, 4, and 6 and IFNG, IL12, and TNF mRNA expression (Table 4 and Fig. 3). No significant correlations were found between TLR1, 2, 4, and 6 and IL6 (Table 4).

Cytokine mRNA expression in bovine luteal cells treated with LPS in culture

Luteal cells were dissociated and cultured for 24h with LPS (0, 0.01, 0.1, 1 μg/ml). Cytokine *(TNF, TGFβ, IFNG, IL6,* and *IL12)* mRNA expression did not significantly differ in response to increasing LPS doses (p>0.05; Fig. 4), even though there were numerical increases observed in *IFNG* and *IL6* mRNA expression with increasing LPS dose. Additional analyses to test for a linear, quadratic, or cubic relationships for cytokine mRNA expression relative to LPS doses were carried out, but did not show any significance (p>0.05).

Discussion:

The major focus of this study was to improve our understanding of role of TLR in the pro-inflammatory/luteolytic process during the estrous cycle in normal animals. However, the data described here may also be viewed from the perspective that in the CL of cows with uterine infections, TLRs may also respond to the direct actions microbial agents, leading to inappropriate inflammatory pathways that may have adverse impacts on the CL, reproductive cyclicity and pregnancy in these animals.

The studies described above provide compelling evidence for the expression of mRNAs for 8 different TLRs (i.e. *TLR1, 2, 3, 4, 6, 7, 8* and *10)* within the bovine CL by PCR analysis. *TLR1-TLR10* mRNAs were also found to be expressed in bovine follicles raising the possibility that TLR mRNA expression continues following ovulation and during luteinization of the follicle into a fully functioning CL (Herath et al. 2007; Price et al. 2013).

Stage IV CL are essentially CL undergoing luteal regression as confirmed by the reduced CL progesterone concentrations seen at this stage, which prompts us to suggest that the increase of *TLR1, 2, 4* and *6* mRNA expression (Figure 1) may be associated with, or in some way involved in, the process of luteolysis potentially through the up-regulation of pro-inflammatory cytokines (*IFNG*, *TNF* and *IL12;* Figure 2). The correlation data (Table 4 and Fig. 3) showing significant positive correlations between *TLR1, 2, 4* and *6* mRNAs and these cytokines, support the notion that these TLRs may function to promote pro-inflammatory cytokine secretion during luteolysis in the cow. Luteolysis has been described as a pro-inflammatory event (Walusimbi and Pate, 2013), and the significantly elevated mRNA expression of pro-inflammatory cytokines *TNF, IFNG* and *IL12* mRNA observed in this study in stage IV CL, support this notion. Numerous studies have demonstrated that TNF and IFNG act as luteolytic, and (cooperatively) as cytotoxic, agents within the bovine CL (Petroff et al. 2001; Skarzynski et al. 2008; Walusimbi and Pate, 2013), and these cytokines have been shown by others to display elevated mRNA expression in regressing bovine CL (Petroff et al. 2001). Furthermore, Neuvians and colleagues (Neuvians et al., 2004) have shown that PGF2α treatment of cows increased *TNF, IFNG*, and *IL1B* mRNA expression, and that *TNF* and *IFNG* treatment of bovine luteal cells in vitro inhibited progesterone production in a dose dependent manner. Interleukin 12 (*IL12)*, which was also elevated in regressing CL in our studies, is a classical pro-inflammatory cytokine, and was shown to promote natural killer cell differentiation, leading to increased *IFNG* mRNA expression (Yang et al. 2011). Since in our studies both *IL12* and *IFNG* were elevated, a similar relationship may also exist in the bovine CL. It is worth considering what ligands might be involved in activating these TLRs in the bovine CL of a normal healthy animal, in the absence of ligands (e.g. LPS) generated by bacteria or viruses. It has been suggested that the breakdown products of luteal tissue and dead or dying cells, such as those of the extracellular matrix, heat-shock proteins and high mobility group box 1 (HMGB1) proteins, may activate TLR (e.g TLR2 and 4) and thus promote pro-inflammatory cytokine production (Kawai and Akira, 2010). Since stage IV represents regressing (i.e. degenerating) CL, it is likely that this would represent a rich source of potential endogenous ligands to activate TLRs expressed within this tissue.

The results presented here for *TLR 2* and *4* are consistent with a recent publication showing that these two *TLR* are expressed in the bovine CL, and that their mRNA expression was increased in CL collected at mid and late stages of the estrous cycle, compared with those collected during the early cycle (Luttgenau et al. 2016). These investigators were also able to demonstrate the mRNA expression of *TLR 2* and *4* at the protein level using immunohistochemistry, and they observed immunoreactivity in both endothelial and luteal cells, providing some critical insights into the cellular location of these *TLRs* (Luttgenau et al. 2016).

Even though it is clear that *TLR 2* and *4* are expressed on luteal and endothelial cells in non-regressing CL (Luttgenau et al. 2016), it is possible that as luteolysis proceeds, at least some of the increased *TLR2* and *4* mRNA’s observed in the regressing (stage IV) bovine CL described above, may be associated with increased immune cell (e.g. macrophages, T-cells, and neutrophils) infiltration into the CL (Walusimbi and Pate 2013). In the present study, *CD45*, which is a common leukocyte marker (Aflatoonian and Fazeli 2008; Kawai and Akira 2010), increased at stage III (vs. stage I), but decreased again at stage IV, while *TLR1*, *2*, *4* and *6* mRNAs were at their maximal levels in CL undergoing luteal regression (stage IV). Thus, while the increased mRNA expression of *CD45* from the developing CL (stage I) to a fully functional CL (stage III) may be explained by the increased infiltration of immune cells into the CL during this period, the decline in *CD45* mRNA observed in stage IV CL, is difficult to explain since this is a time when immune cell infiltration would be maximal (Walusimbi and Pate 2013).

Further evidence to indicate that TLR4 in bovine CL is functional, comes from our data showing that two critical components of TLR4 signaling in response to its ligand LPS, namely *MD2* and *CD14,* were also expressed at mRNA level in the bovine CL. MD2, which is a TLR4 co-receptor, is located found on the external cell membrane and aids in the binding of ligand (e.g. LPS) to the extracellular domain of the TLR4 dimer (Hirata et al. 2005). Cluster of differentiation (CD) 14 also plays a role in the binding of LPS to TLR4, and is an essential component of the functional TLR4 complex (Kawai and Akira 2010). The mRNA expression of *CD14* and *MD2* mRNAs seen in fully functional CL (stage III) were significantly elevated compared with early (stage I) and late (IV) stages, suggesting that these components are expressed prior to the elevated mRNA expression of TLR4 seen at stage IV, to prepare the TLR4-receptor-signaling complex for a critical role in luteal regression.

It should be noted that *TLR2* and *4*, and all cytokine mRNAs were found to be decreased in pregnancy vs. stage IV CL. The general pattern of reduced pro-inflammatory TLR and cytokine mRNA expression profiles during gestation may be needed to prevent luteolysis, and ensure sufficient progesterone secretion to maintain pregnancy and embryo survival (Aflatoonian and Fazeli 2008).

Even though the anti-inflammatory cytokine *TGFB* was expressed in the bovine CL, it did not show a differential mRNA expression patterns based on CL stage. *TGFB* may have a role in the CL throughout the estrous cycle and pregnancy to counter any unexpected inflammatory signals that may threaten CL maintenance, and thus may play a critical role in the ongoing regulation of luteal lifespan. Alternatively, since PGF2α treatment of bovine luteal endothelial cells induced the mRNA expression of TGFB, it has been suggested that TGFB may play a role in disassembly of the microvasculature during luteal regression (Maroni and Davis 2011).

In the current study, the intracellular TLRs (*TLR3*, *TLR7*, and *TLR8*) did not display significantly different mRNA expression profiles at different stages of the estrous cycle or pregnancy. These TLRs recognize viral or bacterial nucleic acids (Kawai and Akira 2010), and given the location of the CL, the likelihood of luteal cells coming into direct contact with bacteria or viruses is probably very small. Thus, the function of these TLR within the CL is unknown and warrants further investigation. However, it has been suggested that DNA arising from dead or dying cells may bind these TLR and activate their intracellular pathways (Kawai and Akira 2010). Thus since luteolysis results in wholesale death of multiple cells types, these TLR may also play an important role within the regressing CL.

In the culture studies, we used dissociated bovine luteal cells from mid-cycle (stage II) CL to examine a functional role of *TLR4*, using its ligand LPS, on cytokine production, as a model for the pro-inflammatory response seen during luteolysis.

However, multiple analyses of linear, quadratic, and cubic relationships between different LPS treatment doses were unable to reveal any statistically significant changes in cytokine mRNA expression, even though some numerical increases in *IFNG* and *IL6* mRNA expression were evident. There are several possible explanations for the apparent lack of an effect of LPS on luteal cell cytokine mRNA expression. Firstly, while the CL used for these studies were stage II, to maximize the yield of viable luteal cells and their survival during the culture, *TLR4* levels (mRNA expression) were quite low at these stages, and only increased in stage IV CL. It is possible that while a more robust response to LPS may be observed in stage IV cells in vitro, obtaining sufficient viable and culture-healthy cells to carry out this study would probably represent an insurmountable challenge. Secondly, the CL is a heterogeneous tissue composed of multiple cell types such as large luteal cells, small luteal cells, fibroblasts, endothelial cells and immune cells (Skarzynski et al. 2008), many of which may express *TLR4* and be targets for LPS (Aflatoonian and Fazeli, 2008; Kawai and Akira 2010; Luttgenau et al. 2016). Variations in the viability or proportion of any of these cell types could contribute to the variability seen in these cultures, which did show some evidence of a numerical increase in *IFNG* and *IL6* at the highest dose, but were not significant. It is worth noting that in recent studies, the effects of LPS on bovine (mid-cycle) CL function were examined in vivo, and decreased enzymes associated with progesterone secretion and elevated *IL1B* and *TNF* mRNA expression were observed (Luttgenau et al. 2016), suggesting that the bovine luteal TLR4 receptor acts as a functional receptor.

In conclusion, we have presented evidence for the mRNA expression of several *TLR*, TLR4 accessory proteins (CD14 and MD2) and cytokine mRNAs in bovine CL, which appear to be maximal in mid (stage III; CD14 and MD2) and late (stage IV; TLR and cytokines) CL. These findings suggest an involvement of one or more of the TLR in the process of luteolysis, and perhaps in the increased mRNA expression of pro-inflammatory cytokines, which also play a role in luteolysis. Finally, in view of the prevalence of infectious diseases such as uterine infections (Sheldon et al. 2009) and mastitis (Barker et al.1998) in dairy cattle, the data presented in this manuscript also raise the possibility that TLR on the bovine CL may mediate adverse effects of infectious pathogens, leading to infertility or sub-fertility.

Acknowledgements:

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

J E Gadsby, IM Sheldon and DH Poole designed the study; AM Tyson, HA Faircloth, M D'Annibale-Tolhurst, J Chang, and PW Farin carried out the study and collected the data;

J E Gadsby, D. H. Poole and A.M. Tyson Nipper, analyzed the data and wrote the manuscript

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FIGURE LEGEND:

Table 1: Primer Sequences for semi-quantitative PCR

Table 2: Primer Sequences for quantitative real-time PCR

Table 3: mRNA expression, via semi-quantitative PCR, of various toll like receptors (TLR3, 7, 8, and 10) and their co-activators, Lymphocyte antigen (MD2) and Cluster of Differentiation (CD) 14, and 45, at various stages of luteal development (I = days 1-4: n=7, II = days 5-10: n=11, III = days 11-17: n=11, IV = days 18-20 of the estrous cycle: n=13, and P = pregnancy: n=9). Data are represented as least squared means± SEM and different letters within a row indicate significant differences (P<0.05), whereas * within a row indicates statistical tendencies (0.1<P>0.05).

Table 4: Correlation table between toll like receptors (TLR) 1, 2, 4 and 6 mRNA expression and proinflammatory cytokines, interferon gamma (IFNG), interleukin (IL) 6, 12, and tumor necrosis factor alpha (TNF), mRNA expression. * indicates statistically significant correlations (P<0.001).

Figure 1: Toll like receptor (*TLR)1, 2, 4 and 6* mRNA expression, via quantitative Real-Time PCR, at various stages of luteal development (I = days 1-4: n=7, II = days 5-10: n=11, III = days 11-17: n=11, IV = days 18-20 of the estrous cycle: n=13, and P = pregnancy: n=9). Expression of *TLR1, 2, 4 and 6* increased in the regressing CL (days 18-20 of the estrous cycle) compared to other stages of the estrous cycle. Data are presented as least-square means ± SEM. Different letters within a specific gene indicate significant differences (P<0.05).

Figure 2: Interferon gamma *(IFNG),* Interleukin (*IL) 6*, *12*, Transforming Growth Factor beta (*TGFB*) and Tumor necrosis factor alpha *(TNF)* mRNA expression, via quantitative Real-Time PCR, at various stages of luteal development (I = days 1-4: n=7, II = days 5-10: n=11, III = days 11-17: n=11, IV = days 18-20 of the estrous cycle: n=13, and P = pregnancy: n=9). Expression of *TNF*, *IFNG,* and *IL12* increased in the regressing CL (days 18-20 of the estrous cycle) compared to other stages of the estrous cycle. Data are presented as least-square means ± SEM. Different letters within a specific gene indicates significant differences ($p<0.05$), whereas * within a specific gene indicates statistical tendencies ($0.1<p>0.05$).

Figure 3: Correlation between toll like receptor (TLR) 4 mRNA expression and proinflammatory cytokines, interferon gamma *(IFNG)*, interleukin (*IL) 6*, *12,* and tumor necrosis factor alpha *(TNF),* mRNA expression. Expression of *TNF*, *IFNG,* and *IL12* displayed a positive relationship with TLR4, whereas this relationship was not observed with IL6 mRNA expression.

Figure 4: Interferon gamma *(IFNG),* Interleukin (*IL) 6*, *12,* tumor necrosis factor alpha *(TNF)* and transforming growth factor beta (*TGFB)* mRNA expression, via quantitative Real-Time PCR, in luteal cells cultured in the presence of either 0, 0.01, 0.1, or 1 μg/ml Lipopolysaccharide (LPS) for 24 hours. No differences were observed in cytokine expression following treatment is increasing concentrations of LPS (n=3). Data are presented as least-square means ± SEM.

Table 1: Primer Sequences for semi-quantitative PCR

Target	**GenBank accession no.**	**Primer Sequence**	**Cycle**	**Amplicon s**
ACTB	NM_173979.3	Fwd 5’ ATC GGC AAT GAG CGG TTC C-3’ Rev 5’ GTG TTG GCG TAG AGG TCC TTG-3’	33	143
CD14	NM_174008.1	Fwd 5’ GGG TAC TCT CTG CTC AAG GAA C-3’ Rev 5’ CTT GGG CAA TGT TCA GCA C-3’	36	199
CD45	AJ400864.1	Fwd 5’ CTC GAT GTT AAG CGA GAG GAA T-3’ Rev 5’ TCT TCA TCT TCC ACG CAG TCT A-3’	36	185
MD2	NM_001046517.1	Fwd 5’ GGG AAG CCG TGG AAT ACT CTA T-3’ Rev 5’ CCC CTG AAG GAG AAT TGT ATT G-3’	36	204
TLR3	NM_001008664.1	Fwd 5’ GAT GTA TCA CCC TGC AAA GAC A-3’ Rev 5’ TGC ATA TTC AAA CTG CTC TGC T-3’	36	195
TLR7	NM_001033761.1	Fwd 5’ TCT TGA GGA AAG GGA CTG GTT A-3’ Rev 5’ AAG GGG CTT AAG GAA TAT C-3’	36	205
TLR8	NM_001033937.1	Fwd 5’ TAA CCT TCG GAA TGT CTC CAG T-3’ Rev 5’ GTG GGA AAT TCT GTT TCG ACT C-3’	36	232
TLR10	NM_001076918.2	Fwd 5’ ATG GTG CCA TTA TGA ACC CTA C-3’ Rev 5’ CAC ATG TCC CTC TGG TGT CTA A-3’	36	239

Table 2: Primer Sequences for quantitative real-time PCR

Target	GenBank accession no.	Primer Sequence	Amplicon
IFNG	NM_174086	Fwd 5’ GAT CTG GAT TCT GAG CCA CTA C-3’	175
		Rev 5’ GCC AGG TAT AAG GTG AGA TGA G-3’	
IL6	NM_173923.2	Fwd 5’ CAA GGA GAC ACT GGC AGA AA-3’	107
		Rev 5’ CAG TGG TTC TGA TCA AGC AAA TC-3’	
IL12A	U14416	Fwd 5’ TCA AGC TCT GCA TCC TTC TTC-3’	254
		Rev 5’ GGT TAT GAG AGA CCT CAG CAT TC-3’	
RPL19	NM_001040516	Fwd 5’ ATC GAT CGC CAC ATG TAT CA-3’	227
		Rev 5’ GCG TGC TTC CTT GGT CTT AG-3’	
TGFB	M36271	Fwd 5’ CGT CAG CTC TAC ATT GAC TTC C-3’	205
		Rev 5’ GGA CCT TGC TGT ACT GTG TAT C-3’	
TNF	NM_173966	Fwd 5’ TCT ACT CAC AGG TCC TCT TCA G-3’	235
		Rev 5’ GAT GTT GAC CTT GGT CTG GTA G-3’	
TLR1	NM_001046504.1	Fwd 5’ ATT TCT TGC CAC CCT ACT CTG-3’	100
		Rev 5’ GTT GAG ACA TGT TGC CAA ACT C-3’	
TLR2	NM_174197.2	Fwd 5’ GCA CTT CAA CCC TCC CTT TA-3’	127
		Rev 5’ GTT CTC CGA AAG CAC AAA GAT G-3’	
TLR4	NM_174198.6	Fwd 5’ TCT ACT GCA GCC AGG ATG AA-3’	92
		Rev 5’ GTA GTG AAG GCA GAG CTG AAA-3’	
TLR6	NM_001001159.1	Fwd 5’ GAC TCT CAA GCA TTT AGA CCT CTC-3’	146
		Rev 5’ GCA AGT GAG CAA TGG GTA GTA-3’	

Table 3:

	Stage of Luteal Development					
Target	**I**	**II**	**III**	**IV**	**P**	**P Value**
CD14	24.6±6.1^{ab}	20.4±3.0^{ab}	32.4±5.9^{ab}	16.1±4.5^{a}	22.5±8.2^{ab}	0.0074
CD45	16.7±2.1^{a}	23.2±2.6^{ab}	31.3±4.4^{b}	17.5±4.0^{a}	25.0±4.2^{ab}	0.0036
MD2	13.2±1.6^{a}	10.4±1.4^{a}	21.5±3.3^{b}	13.0±3.1^{a}	10.5±2.5^{a}	0.0376
TLR3	39.2±11.9	40.6±2.3	42.8±5.3*	26.8±2.8*	40.1±3.6	0.082
TLR7	10.4±1.6	11.7±1.5	16.4±7.8	14.3±12.9	10.5±6.8	>0.05
TLR8	10.9±2.2	15.5±5.0	18.6±2.3	22.8±8.0*	9.8±3.1*	0.084
TLR10	14.4±3.2	8.3±2.9	8.7±4.2	9.3±7.0	10.1±2.7	>0.05

Table 3: mRNA expression, via semi-quantitative PCR, of various toll like receptors (TLR3, 7, 8, and 10) and their co-activators, Lymphocyte antigen (MD2) and Cluster of Differentiation (CD) 14, and 45, at various stages of luteal development (I = days 1-4: n=7, II = days 5-10: n=11, III = days 11-17: n=11, IV = days 18-20 of the estrous cycle: n=13, and P = pregnancy: n=9). Data are represented as least squared means± SEM and different letters within a row indicate significant differences (P<0.05), whereas * within a row indicates statistical tendencies (0.1<P>0.05).

Table 4:

	Proinflammatory Cytokines			
	IFNG	IL6	IL12	TNF
TLR1	0.77*	-0.02	0.75*	0.78*
TLR2	0.71*	-0.01	0.70*	0.75*
TLR4	0.61*	0.09	0.60*	0.65*
TLR6	0.80*	0.05	0.77*	0.80*

Table 4: Correlation table between toll like receptors (TLR) 1, 2, 4 and 6 mRNA expression and proinflammatory cytokines, interferon gamma (IFNG), interleukin (IL) 6, 12, and tumor necrosis factor alpha (TNF), mRNA expression. * indicates statistically significant correlations ($P<0.001$).

Figure 1

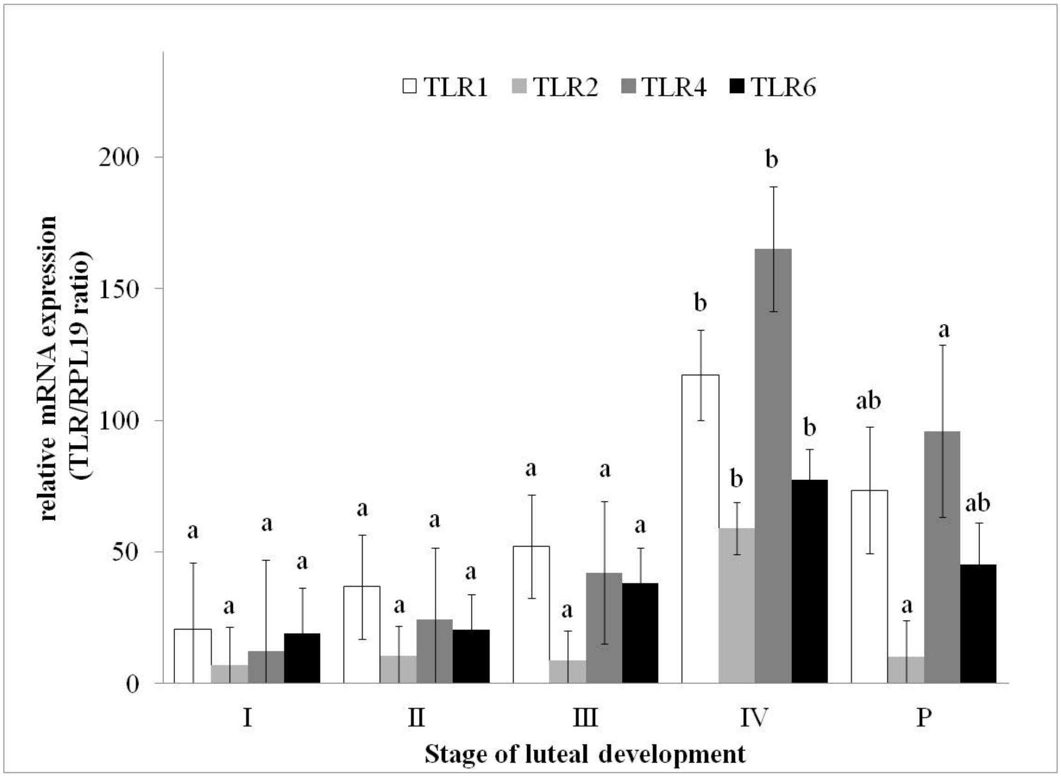

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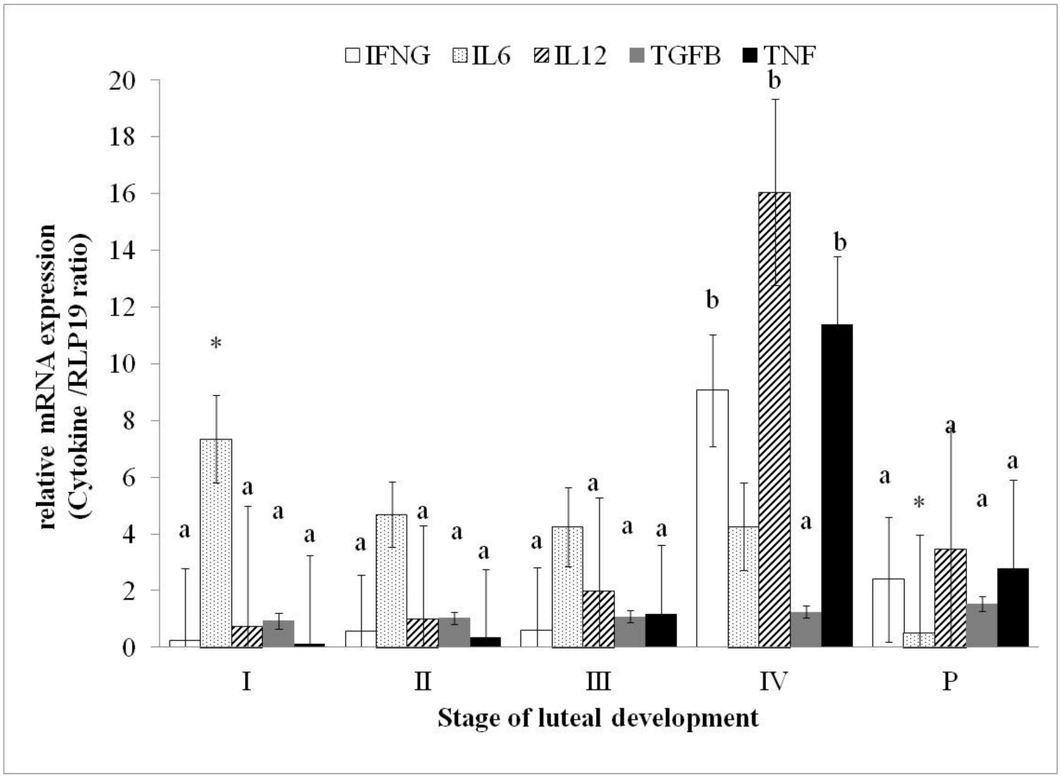
Figure 2:



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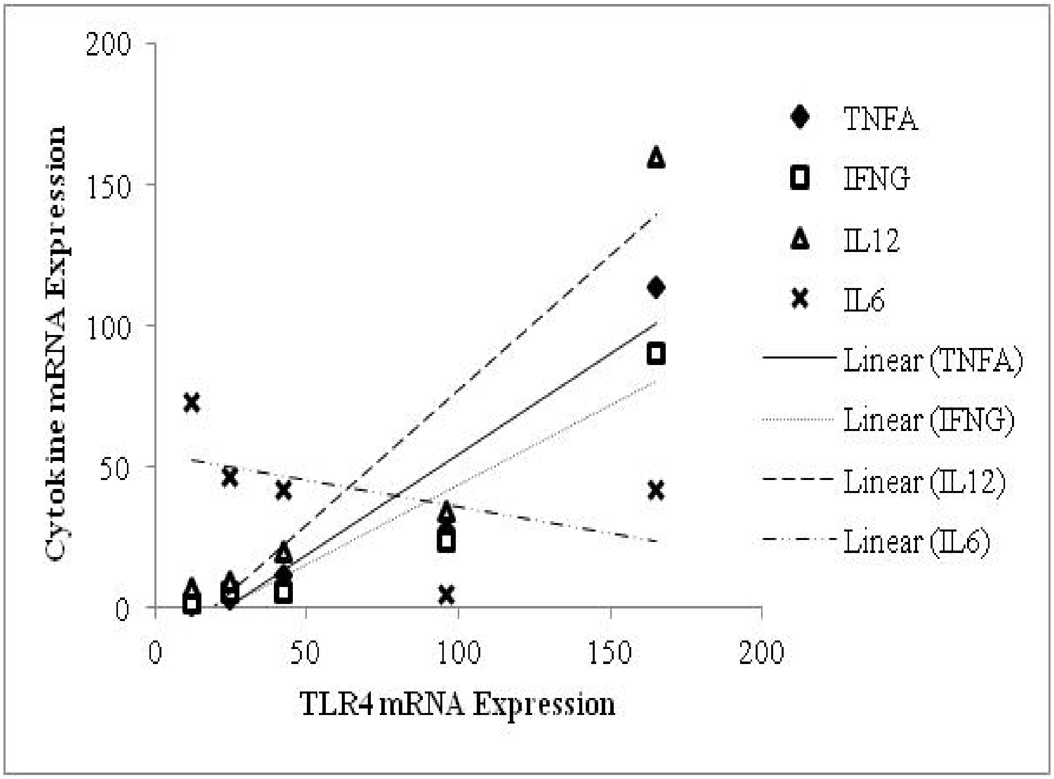

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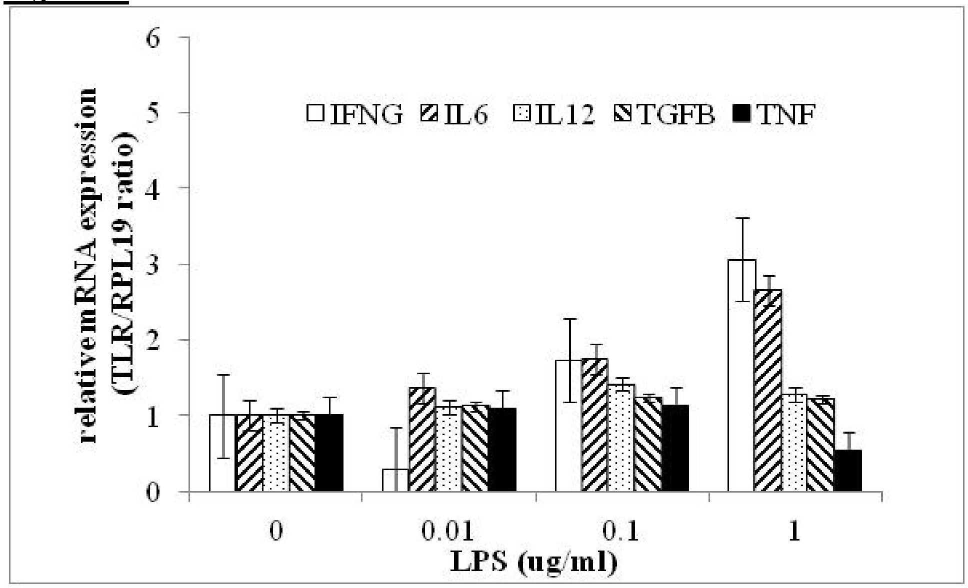

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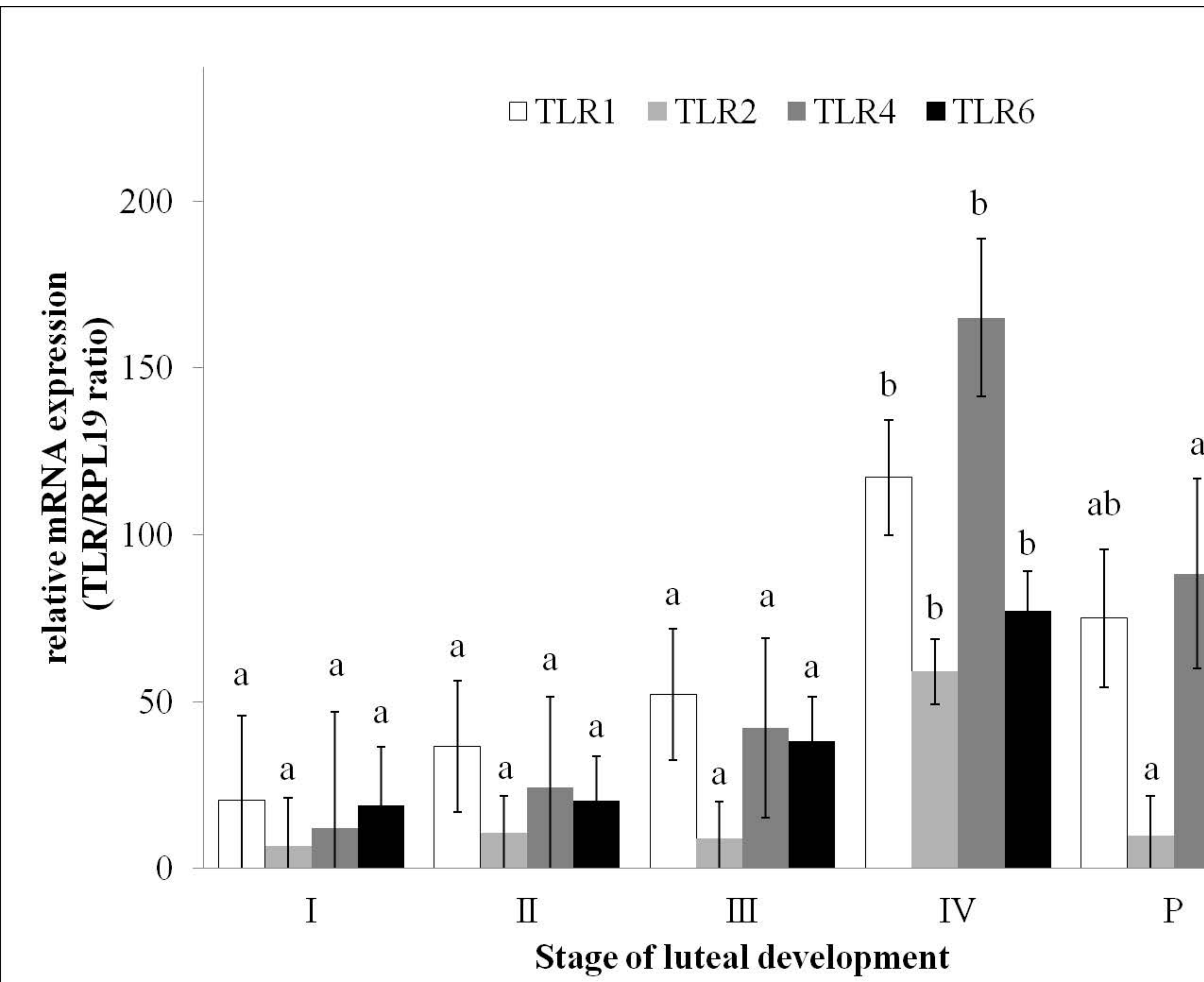
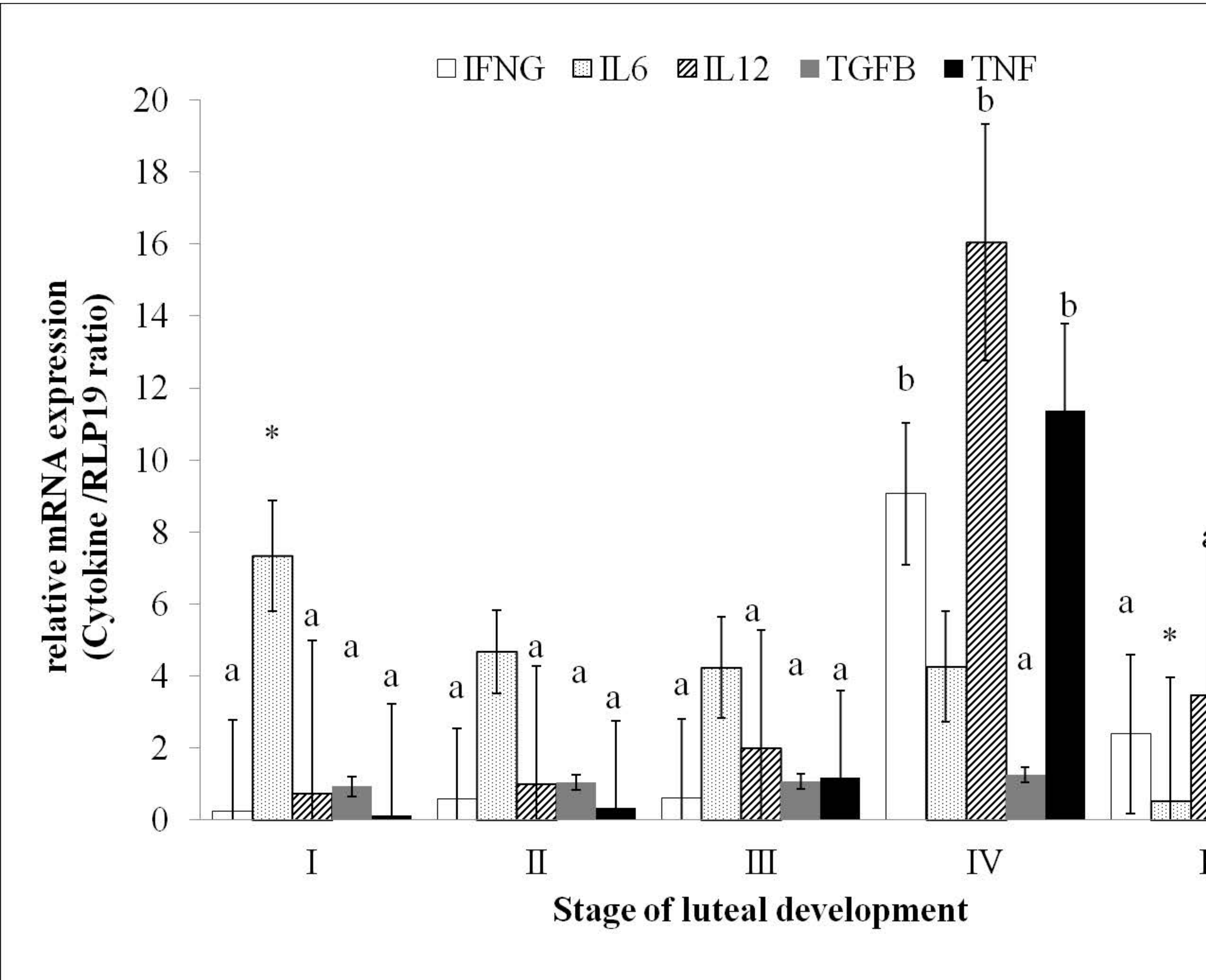
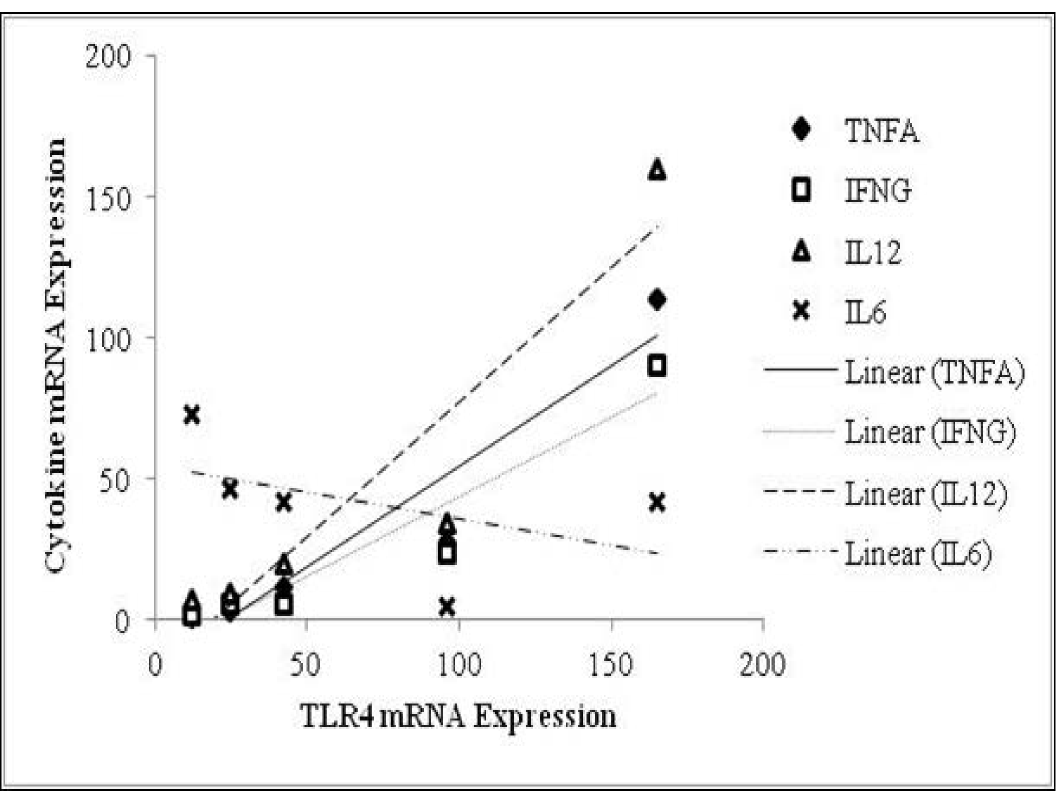
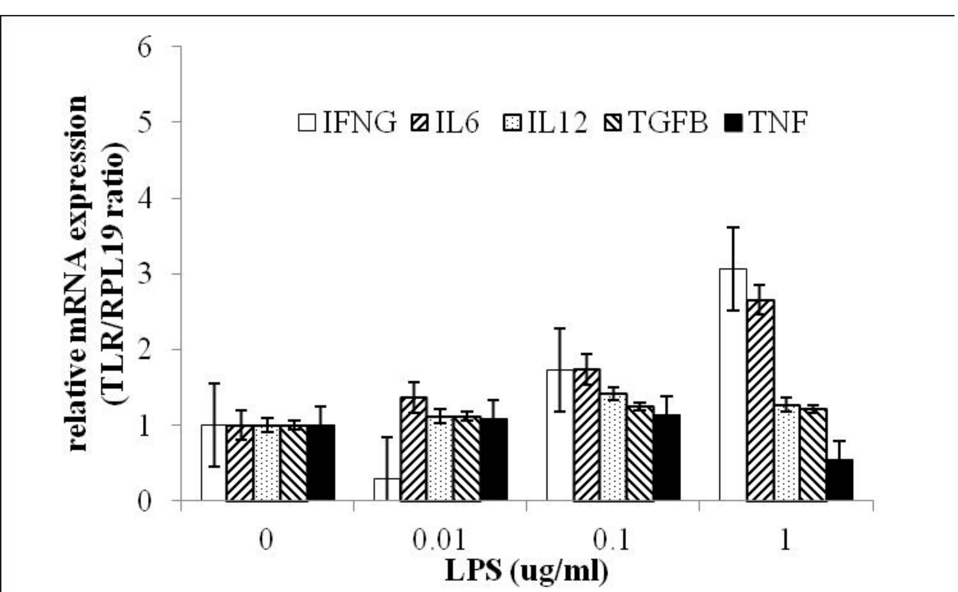

TLR1 TLR2 TLR4 TLR6
relative mRNA expression (TLR/RPL19 ratio)
200
150
100
50
0
a
b
ab
I
II
III
IV
P
Stage of luteal development



IFNG
IL6
IL12
TGFB
TNF
20
18
16
14
12
10
8
6
4
2
0
relative mRNA expression
(Cytokine /RLP19 ratio)
I
II
III
IV
Stage of luteal development



200
150
100
50
0
0
50
100
150
200
Cytokine mRNA Expression
TLR4 mRNA Expression
TNFA
IFNG
IL12
IL6
Linear (TNFA)
Linear (IFNG)
Linear (IL12)
Linear (IL6)



6
5
4
3
2
1
0
IFNG IL6 IL12 TGFB TNF
relative mRNA expression (TLR/RPL19 ratio)
0
0.01
0.1
1
LPS (ug/ml)