



Toll-like receptor 2 and 4 expression in the bovine corpus luteum during the different stages of the estrous cycle

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Abstract

The aim of this study was to elucidate the presence of components of the innate immune system in the bovine corpus luteum (CL) by detecting the expression and cell-specific localization of TLR2 and TLR4 during different stages of the estrous cycle in a control study design. Bovine CL samples were collected from a local slaughterhouse and assigned to three groups as follows: developing CL (dCL; n = 6, approx. days 3–6), mature CL (mCL; n = 5, approx. days 8–12), and regressing CL (rCL; n = 5, approx. days 17–19). An upregulation of TLR2 mRNA was detected only in rCL ($P < 0.05$). Localization of the TLR2 protein was particularly apparent in luteal cells and a prominent immunofluorescent signal corresponding to TLR2 was detected only in rCL. TLR4 mRNA were higher in mCL and rCL compared to dCL ($P < 0.05$). The presence of the TLR4 protein in bovine CL was clearly detected in the luteal cells of both mCL and rCL. The results of this study suggest a role for TLRs in the development, maintenance, and regression of bovine CL. TLR signaling mediated pathway in luteal cells may involve in the regression of CL via regulation of TLR2 and TLR4.

Key words: bovine, corpus luteum, toll-like receptors.

Introduction

The corpus luteum (CL) forms after ovulation from remaining Graafian follicular cells and secretes progesterone, which has vital functions for the establishment and maintenance of pregnancy in many species, including cows. If a pregnancy does not occur, the CL is required to regress in order to allow the cow to return to estrus. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), released by the non-pregnant uterus, initiates regression of the CL in the cows (Pate *et al.*, 2012). The CL is composed of multiple cell types, including steroidogenic (large luteal cells and small luteal cells) and non-steroidogenic cells (endothelial cells, pericytes, fibrocytes and immune cells; Wiltbank 1994) and this mixture of cells altogether contribute to the formation and demise of the CL. For example, despite the fact that the $PGF_{2\alpha}$ receptor was only detected in large luteal cells where the first luteolytic signal was expected to start (Mamluk *et al.*, 1998), our previous studies have shown that luteolytic induction occurs in endothelial cells

earlier than in luteal cells (Atli *et al.*, 2011; Atli *et al.*, 2012). Although the role of immune cells in the CL was previously thought to be in phagocytosis following luteolysis (reviewed by Pate and Landis Keyes, 2001), the immune cell population and chemokines in the CL are affected during functional luteolysis in the early response to $PGF_{2\alpha}$ (Shirasuna *et al.*, 2012). Moreover, immune system roles were demonstrated in the dCL in a previous study (Poole and Pate 2012). These results indicate that both the development and regression of the CL are regulated by complex mechanisms.

Toll-like receptors (TLRs), as transmembrane proteins, are an important part of the innate immune system and play roles in the activation of the acquired immune system (Akira, 2003). In addition to their roles in pathogen recognition, TLRs interact with secreted or released endogenous molecules such as reactive oxygen species (ROS), heat shock proteins, and proteins released from dying cells (see review by Piccinini and Midwood 2010). Therefore, activation of TLRs can induce both physiological and pathological processes, including pro/anti-inflammatory processes (Kannaki *et al.*, 2011). In the reproductive system, TLRs participate in ovulation, fertilization, placental function, trophoblast invasion, parturition, and protection of the reproductive tract from pathogens (Horne *et al.*, 2008; Kannaki *et al.*, 2011; Koga and Mor 2008; Silva *et al.*, 2010). They also allow communication between many other components of the immune system (Koga and Mor 2008). TLRs are generally expressed by different types of immune cells, but their expression is also detected in cells of reproductive tissues, such as granulosa cells, endometrial luminal and stromal cells, trophoblasts, and cervical cells (Horne *et al.*, 2008; Koga and Mor 2008). In bovine endometrium, TLRs play roles in the prevention of endometrial infection with microorganisms as they are expressed on epithelial cells of the endometrium (Davies *et al.*, 2008). When TLR signaling is induced by appropriate signal molecules, many downstream targets of TLRs, including IL4, IL8, COX2, and $TNF\alpha$, are regulated (Horne *et al.* 2008; Silva *et al.* 2010). Since the expression of many pro/anti-inflammatory molecules is affected during formation and regression of the CL (Skarzynski and Okuda 2010), the expression of TLRs may play critical roles in regulating the changing physiology of the CL. TLR2 and TLR4 are the best characterized TLRs regarding their signaling mechanisms and are mediated by many inflammatory pathways and endogenous

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substances. Recently, in the CL, the expression of TLR2 and TLR4 was predominantly localized to luteal cells and blood vessels and increased during the mid- (Lüttgenau *et al.*, 2016) and late luteal phase (Lüttgenau *et al.*, 2016; Gadsby *et al.*, 2017). Therefore, the aims of the present study were to approve the recent findings on 1) the expression of TLR2 and TLR4 during different stages of the estrous cycle, and 2) on cell-specific localization of TLR2 and TLR4 in the bovine CL.

Materials and Methods

CL tissue collection

Bovine CL samples were collected from a local slaughterhouse in Konya, Turkey. Genital tracts, including ovaries, were obtained within 10 minutes after slaughter and immediately transferred to the laboratory on ice. Genital tracts were washed with PBS and classified based on the criteria mentioned by Arosh *et al.* (2002) that characterized the stage of the estrous cycle through morphology of the ovarium and uterus. A further classification for the collected bovine CLs was carried out by examining the expression of StAR mRNA and protein. The CLs were removed and assigned to three groups as follows: developing CL (dCL; $n = 6$, approx. days 3–6), mature CL (mCL; $n = 5$, approx. days 8–12), and regressing CL (rCL; $n = 5$, approx. days 17–19). All animal material collection procedures were approved by the Ethics Committee of Dicle University, Diyarbakir, Turkey. The CLs were dissected, snap-frozen in liquid nitrogen, and stored at -80°C for later isolation of RNA. A portion of CL tissue was fixed in a 4% paraformaldehyde solution overnight at 4°C and then dehydrated using serial dilutions of methanol (25%, 50%, 75%, and 100%). Samples were stored in methanol at -20°C until evaluated by immunofluorescence.

RNA isolation, RT reaction, and Quantitative PCR

About 20 mg of CL tissue was homogenized in Trizol[®] (Invitrogen, Carlsbad, CA, USA) using a homogenizer (Slientcruzer M, Heidolph, Germany). Total RNA was isolated according to the manufacturer's protocol. The RNA integrity was evaluated by agarose gel (1.5%) electrophoresis and optical density measurements at 260/280 nm of 2 ± 0.1 with a NanoDrop ND-2000 (Thermo Scientific, Wilmington, DE, USA). Two micrograms of RNA were incubated with DNase I (Fermentas Vilnius, Lithuania) to eliminate possible genomic DNA contamination and then the RNA was reverse transcribed in the presence of oligo-dT primers using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas Vilnius, Lithuania) according to the manufacturer's protocol. To clarify RT specificity, analyses were also performed with all components of the RT kit without including RNA (RT negative). Oligonucleotide primers for StAR, TLR2, TLR4, and GAPDH were designed using Primer3 from the sequences in the NCBI gene database or from published primer sequences (Atli *et al.*, 2012). The primer pair sequences and product sizes are shown in

Table 1. All PCR reactions were set up as follows: 5 μL SYBR Green Master Mix (Maxima[™] SYBR, 2X, Fermentas Vilnius, Lithuania), 2.5 pMol of each primer, 0.5 μL cDNA, and ddH₂O to bring the final volume to 10 μL . Thermal cycling was performed by incubating the mixture at 50°C for 2 min with subsequent denaturation at 95°C for 10 min. This was followed by 40 cycles of denaturation, annealing, and amplification (95°C 30 sec, 60°C 30 sec, 72°C 30 sec). All reactions were performed on an Applied Bioscience Stepone plus Real-Time PCR System (Foster City, CA, USA). Melting curve analyses were performed as follows: 95°C for 1 min followed by fluorescence measurements performed at 1.5°C increments between 60°C and 95°C . In each run, negative controls without a cDNA template and RT negative controls were included. To verify reaction specificity, amplification products were evaluated by separation on agarose gel. All samples were evaluated in duplicate for each cDNA. From RNA extraction to real-time PCR, were performed twice as technical replicates. Amplifications were performed on a serial dilution of pooled cDNA to determine the primer efficiency. The qPCR amplification efficiency for specific gene amplicons was similar.

Table 1. List of primer used in real-time PCR.

Genes	Primers	Size
StAR	F 5' -cagcagaagggtgtcatcaga-3'	152
	R 5' -gagaggacctggtgatgatg-3'	
TLR2	F 5' -ggtttaaggcagaatcggttg-3'	190
	R 5' -aaggcaactgggttaaactgtgt-3'	
TLR4	F 5' -tgctggctgcaaaaagtatg-3'	149
	R 5' -cctgtagtgaaggcagagc-3'	
GAPDH	F 5' -aatatcatcctgcttctactgg-3'	154
	R 5' -cactctggcaggtttctcca-3'	

Immunofluorescence

Tissue sections were deparaffinized in xylene, rehydrated, and boiled in 10 mM sodium citrate (pH 6.0) for 20 min. Tissues were washed with a solution containing Tris-HCl (25 mM pH 7.5), NaCl (140 mM), KCl (2.7 mM), and 0.1% Tween-20 (TBSTw) and non-specific binding sites were blocked for 1 hr in a blocking solution (TBSTw containing 1% Blocking Reagent (Roche Diagnostics, Indianapolis, IN), normal goat sera (for TLR4) or normal human serum (for TLR2), and 1% bovine serum albumin. Tissues were incubated overnight at 4°C with primary antibodies diluted in the blocking solution as follows: 1:100 rabbit anti-StAR (sc-25806, Santa Cruz Biotech, polyclonal), 1:100 rabbit anti-TLR4 (AA 780-830, antibodies-online.com, polyclonal), 1:150 human anti-BovineTLR2 (CD282 Antibody, AbD Serotec, Bio Rad, USA, monoclonal). After several washes with TBSTw, tissues were incubated for 1 hr with the blocking solution containing 1:250 Dylight488-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) (for TLR4, StAR) and 1:250 mouse anti human: Fluorescein isothiocyanate (FITC, for TLR2, AbD Serotec, Bio Rad, USA). Auto fluorescence was also blocked by Autofluorescence Blocking Solution

containing 10 mM Cu(II)SO₄ and 50 mM NH₄Ac. For negative controls, sections were incubated by secondary antibodies or isotype control. Labeled tissue sections were counterstained with antifade mounting medium with DAPI (Vectashield, Vector Lab, USA). Fluorescence images were captured using a Nikon Eclipse E600 compound microscope (Nikon Instruments Inc., Melville, NY).

Statistical analysis

Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used to normalize the expression of target genes. The use of GAPDH as a house-keeping gene has been previously validated (Atli *et al.*, 2012). Furthermore, we also did validate the consistency of steady-state *GAPDH* concentrations in the different stages of CLs. Before statistical analysis, data normalization was performed with the necessary assumptions according to Livak and Schmittgen (2001)

via the $2^{-\Delta Ct}$ method. Non-parametric tests were applied. Comparisons between the different stages of the estrous cycle were carried out using Kruskal–Wallis tests and the Mann–Whitney U-test was applied post hoc. Data were considered statistically significant when P values were lower than 0.05.

Results

Expression of *StAR* mRNA and protein in collected corpus luteum samples

An important component of progesterone production, *StAR* mRNA and protein expression were evaluated in collected corpus luteum samples. While expression of *StAR* mRNA was upregulated between dCL and mCL, it was dramatically downregulated in rCL (Fig. 1). Moreover, protein localization of *StAR* in luteal cells also decreased to an undetectable level on microscopy observation in rCL (Fig. 2).

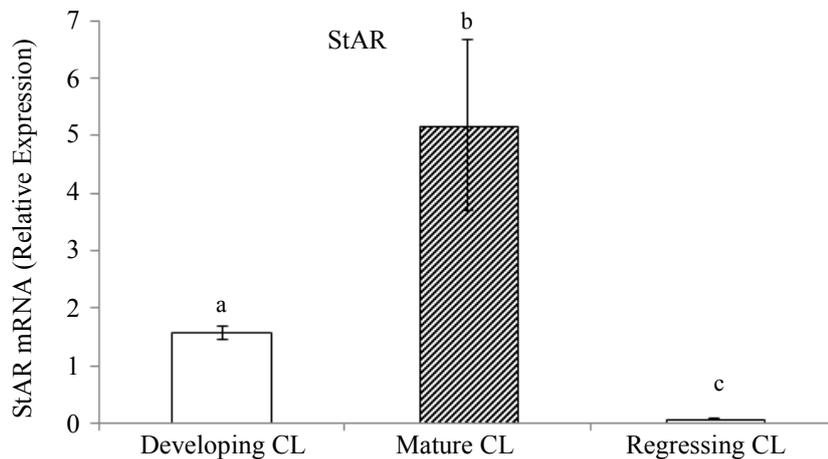


Figure 1. Relative expression of *StAR* mRNA (Mean ± SEM) in the bovine CL during the different stages of the estrous cycle; developing CL (dCL; n = 6, approx. days 3-6), mature CL (mCL; n = 5, approx. days 8-12), and regressing CL (rCL; n = 5, approx. days 17-19) The different letter (a, b, c) indicates differences ($P < 0.05$) among groups.

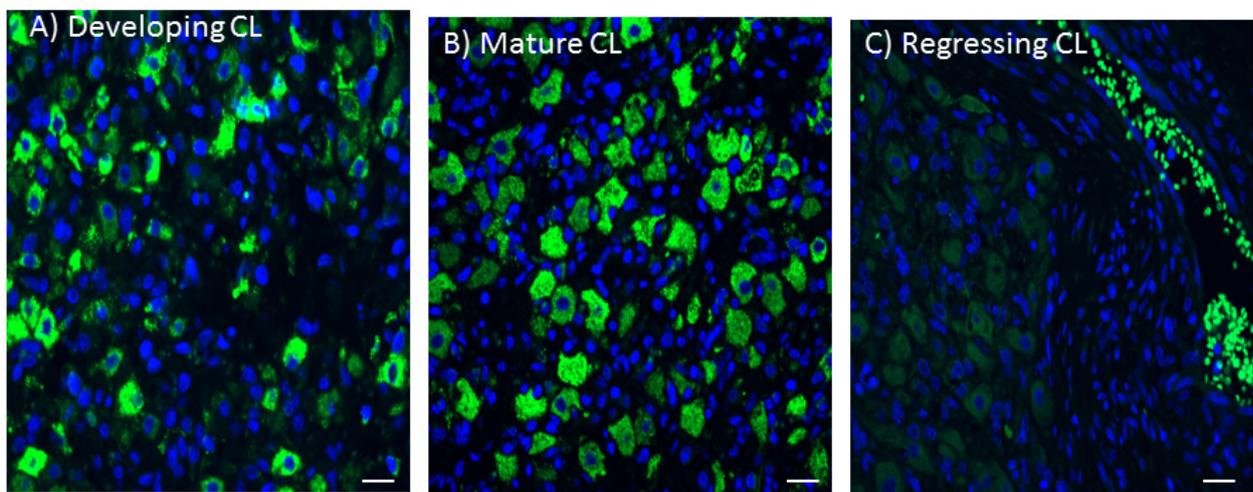


Figure 2. Representative images of immunofluorescence staining for *StAR* in developing CL (A) mature CL (B), regressing CL (C). Nuclei was stained with 4',6-diamidino-2-phenylindole dilactate (DAPI), scale bar represent 50 micrometer.

The effect of the stage of the estrous cycle on TLR2 and TLR4 mRNA expression in the bovine CL

PCR amplified products for TLR2 and TLR4 were the predicted size (data not shown). Relative steady state levels of TLR2 and TLR4 mRNAs in CL samples taken during the different stages of the estrous

cycle, including developing, mature, and regressed stages of the CL, are shown in Figures 3 and 4. Expression of TLR2 and TLR4 mRNAs was detected in all stages of cyclic CLs. An upregulation of TLR2 mRNA was only detected in rCL (Fig. 3). The steady state level of TLR4 mRNA was higher in mCL and remained at high levels in rCL, relative to dCL (Fig. 4).

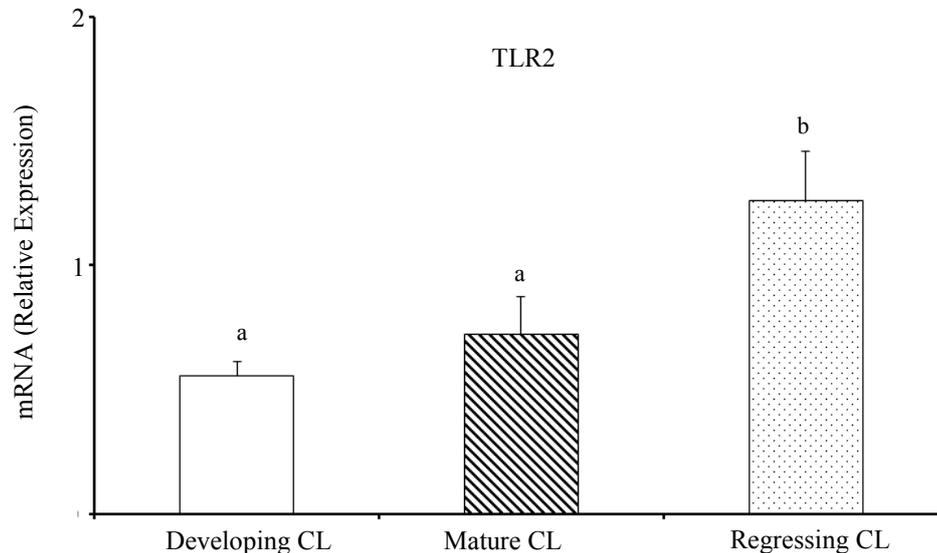


Figure 3. Relative expression of TLR2 mRNA (Mean ± SEM) in the bovine CL during the different stages of the estrous cycle; developing CL (dCL; n = 6, approx. days 3-6), mature CL (mCL; n = 5, approx. days 8-12), and regressing CL (rCL; n = 5, approx. days 17-19) The different letter (a, b) indicates differences ($P < 0.05$) among groups.

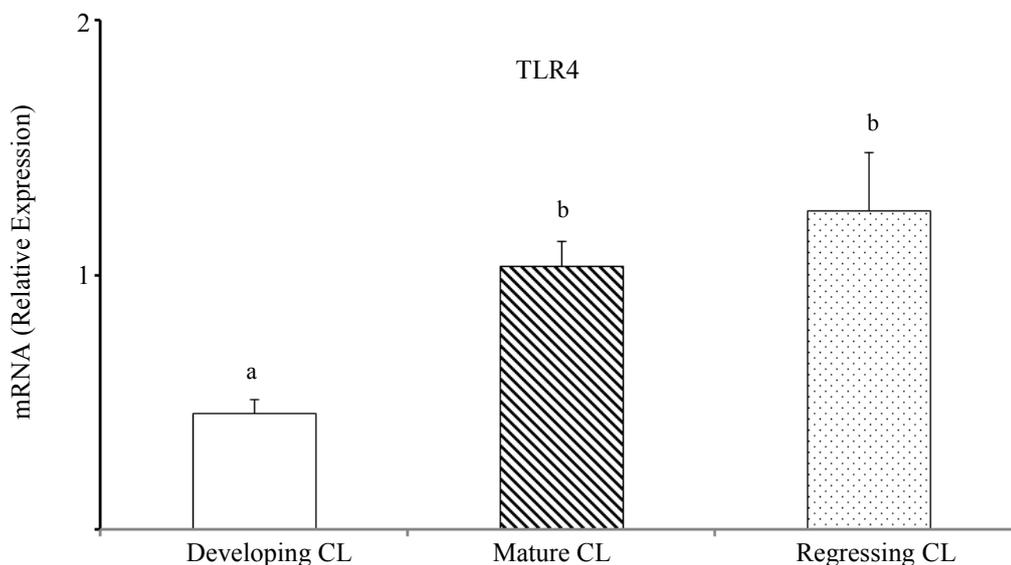


Figure 4. Relative expression of TLR4 mRNA (Mean ± SEM) in the bovine CL during the different stages of the estrous cycle; developing CL (dCL; n = 6, approx. days 3-6), mature CL (mCL; n = 5, approx. days 8-12), and regressing CL (rCL; n=5, approx. days 17-19) The different letter (a,b) indicates differences ($P < 0.05$) among group.

Localization of TLR2 and TLR4 proteins in the bovine CL

The presence of TLR2 protein in bovine CL is shown in Figure 5. Localization of TLR2 protein was apparent in both large and small luteal cells. When

comparing the stages of the estrous cycle, a prominent immunofluorescent signal for the TLR2 protein was observed only in rCL. The presence of the TLR4 protein in bovine CL was clearly detected in luteal cells (Fig. 6) of both mCL and rCL but not dCL.

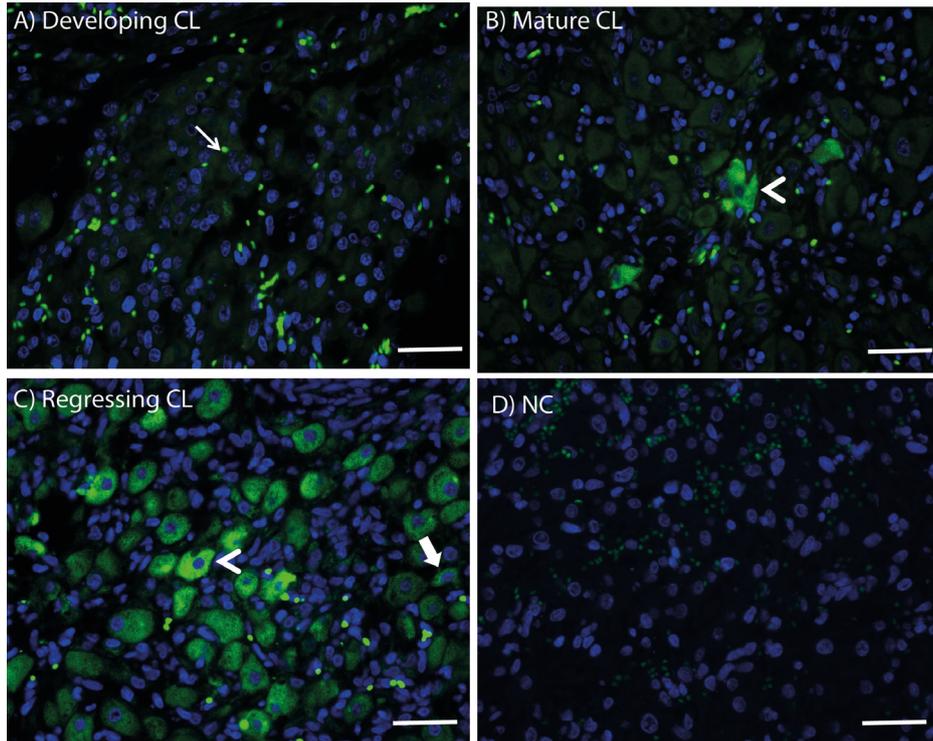


Figure 5. Representative images of immunofluorescence staining for TLR2 (Toll-like receptor 2) in developing CL (A) mature CL (B), regressing CL (C) and Negative Control (NC, D). TLR2 proteins (FITC, 488 nm) were localized in both large (white arrowhead) and small luteal (white large arrow) cells. Erythrocytes (white small arrow) in the CL were also appeared due to auto-fluorescence. Nuclei was stained with 4',6-diamidino-2-phenylindole dilactate (DAPI), scale bar represent 50 micrometer.

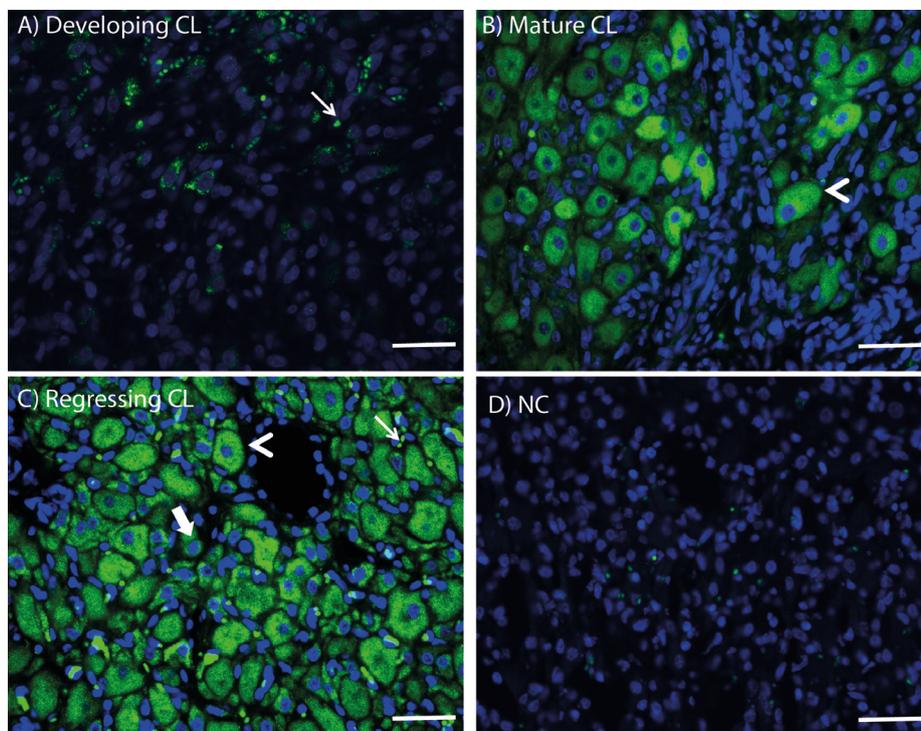


Figure 6. Representative images of immunofluorescence staining for TLR4 (Toll-like receptor 4) in developing CL (A), mature CL (B), regressing CL (C) and Negative Control (NC, D). TLR4 proteins (Dylight488, 488 nm) were localized in both large (white arrowhead) and small luteal (white large arrow) cells. Erythrocytes (white small arrow) in the CL were also appeared due to auto-fluorescence. Nuclei was stained with 4',6-diamidino-2-phenylindole dilactate (DAPI), scale bar represent 50 micrometer.



Discussion

The role of TLR in the reproductive tract has focused on the endometrium, and placental tissues under physiological and pathological conditions such as ovulation, fertilization, LPS challenges, endometritis, chorioamnionitis, and pre-eclampsia (Amjadi *et al.*, 2014; Kannaki *et al.*, 2011). The TLR-regulation of embryonic interferon- τ secretion was clearly shown in the ruminants (Ruiz-Gonzalez *et al.*, 2015). Moreover, during early pregnancy, expression profiles of TLRs dramatically changed in both peripheral blood leukocytes (PBLs) and trophoblast cells of ewes (Kaya *et al.*, 2017). The CL could be another potential tissue for TLR regulation since critical actors of the immune system play roles in the development and regression mechanisms of the CL. Expression profiles and cellular localization of TLR2 and TLR4 were detected in the bovine CL in the present study. Consistent with our results, two different research groups have also published their results which emphasized regulatory roles of TLR2 and TLR4 in the bovine CL during the estrous cycle (Gadsby *et al.*, 2017; Lüttgenau *et al.*, 2016). For example, Lüttgenau *et al.* (2016) indicated TLR 2 and 4 mRNA expressions were upregulated in the CL at mid-and late stages of the estrous cycle, compared with those collected during the developing stage of CL in the estrous cycle. Although Lüttgenau *et al.* (2016) did not show an increase in TLR2 and TLR4 mRNA on day 17 compared to mid stage of the cycle, we report an upregulation for TLR2 in regressing CL of the estrous cycle. A further increase in TLR2 on regression stage of CLs during the estrous cycle in bovine corpus luteum in the present study could be based on using later days CL (on day 17- 19) for our regression group. Similarly, Gadsby *et al.* (2017) have also indicated that an increased expression profiles of TLR2 and TLR4 at mRNA levels during the regression stage of the estrous cycle in bovine CL (days 18–20 of the estrous cycle) collected from the abattoir.

Earlier studies indicated that the luteolytic process was accompanied by changes in the levels of many endogenous molecules including ROS, endothelial-derived proteins, and lipophilic molecules in rCL (Pate *et al.*, 2012; Skarzynski and Okuda 2010). It is also known that these endogenous molecules serve as ligands to induce TLR2 and TLR4 signaling (Piccinini and Midwood 2010; Kannaki *et al.*, 2011). When the cell surface TLRs are bound with endogenous ligands triggering TLR signaling, MyD88 (myeloid differentiation primary response gene 88) -dependent or -independent pathways are activated in the cytoplasm (Fitzgerald *et al.*, 2001). Both pathways stimulate the secretion of pro-inflammatory cytokines. Similarly, pro-inflammatory gene expression increases during regression of the CL (Atli *et al.*, 2012; Shirasuna *et al.*, 2012). Therefore, an increase in both TLR2 and TLR4 mRNA and protein levels in rCL in the present study may indicate roles of TLR signaling in the pro-inflammatory cytokine-mediated regressing mechanism in bovine CL. Similarly, this statement was also confirmed in bovine CL by Gadsby *et al.* (2017). They

are indicated that one or more of the TLRs found within the CL may play a role in luteolysis, perhaps via pro-inflammatory cytokine mRNA expression.

In the present study, expression of TLR2 and TLR4 proteins was detected in luteal cells and may suggest that luteal cells, accompanied by other types of cells, induce the immune system for luteal regression. Similarly, Lüttgenau *et al.* (2016) observed immunoreactivity for TLR 2 and 4 in both endothelial and luteal cells in bovine CL. Earlier studies suggest that CL physiology is closely associated with immune cells at certain stages of the estrous cycle. Specifically, during luteolysis, immune cell infiltration to the CL increases (Penny, 2000) and they are considered to play a role in luteolysis. Although these immune cells are not responsive to PGF_{2 α} in dCL, luteal cells respond to PGF_{2 α} by increasing the expression of those genes that have regulatory functions on immune cells in mCL (Mondal *et al.*, 2011). Therefore, the stimulation of TLR expression in luteal cells in mCL and rCL as observed in the present study may relate to an increased active immune status of the CL. As the immune cells and their signaling molecules have been linked to progesterone production and its regulation (Liptak *et al.*, 2005; Mondal *et al.*, 2011), the progesterone-producing luteal cells may undergo immune-cell like changes to cooperate in the mechanism of luteal maturation and regression.

Bovine CL, during the developing stages, does not respond to luteolytic PGF_{2 α} injection (Mondal *et al.*, 2011). One of the reasons for this non-responsiveness was proposed in a recent study (Sen *et al.*, 2006) in which expression levels of PKC ϵ were found to increase 10-fold in mCL compared to dCL. Moreover, a specific PKC ϵ inhibitor blocked the luteolytic response to PGF_{2 α} in steroidogenic cells (Sen *et al.*, 2006). Similar to PKC ϵ expression in bovine CL, both TLR4 mRNA and protein levels increased in mCL and rCL compared to the developing stages in the present study. Since PKC ϵ is a critical component of TLR4 signaling (McGettrick *et al.*, 2006) and expression of both PKC ϵ and TLR4 increased at the same stages of the CL, TLR4 is possibly a critical component for the PGF_{2 α} response in the CL, implying a potential role of TLR4 in the acquisition of luteolytic capacity. Similarly, immune related genes increased only after PGF_{2 α} induction in mCL compared to dCL (Mondal *et al.*, 2011). Moreover, enhanced populations of specific types of immune cells and cell-to-cell communication between immune and the other types of CL cells in the mature stage clearly indicate immune regulation of the luteolytic pathway (Poole and Pate 2012; Penny 2000).

As a conclusion, the expression levels and cellular localization of TLR2 and TLR4 in bovine CL at different stages of the estrous cycle were described in the present study. Consistent with recently published studies investigating TLR2 and 4 in the bovine CLs (Gadsby *et al.*, 2017; Lüttgenau *et al.*, 2016), our data suggest the possible involvement of these TLRs and a related pathway in luteal cells during the regression period of bovine CL as indicated by increased expression levels of TLR2 and TLR4.



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