

Regulation of Innate Immune Function in Bovine Oviduct Epithelial Cells in Culture: The Homeostatic Role of Epithelial Cells in Balancing Th1/Th2 Response

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Abstract. This study aimed to investigate the role of epithelial cells in regulating innate immunity in bovine oviduct epithelial cell (BOEC) culture. We studied the effect of *Escherichia coli* lipopolysaccharide (LPS) and its interaction with ovarian steroids, estradiol (E2) and progesterone (P4), and luteinizing hormone (LH) at concentrations observed during the preovulatory period on immune responses in BOEC culture. Immunohistochemistry of oviduct tissue showed intensive expression of Toll-like receptor-4 (TLR-4) and TLR-2 in epithelial cells. A dose of 10 ng/ml LPS stimulated *TLR-4*, cyclooxygenase-2 (*COX-2*), nuclear factor kappa B inhibitor A (*NFKBIA*), interleukin 1 β (*IL-1 β*) and tumor necrosis factor α (*TNF- α*) expression, indicating an early pro-inflammatory response. A dose of 100 ng/ml LPS did not induce expression of these genes but stimulated *TLR-2*, *IL-10*, *IL-4* and microsomal prostaglandin E synthase-1 (*mPGES-1*) expression and PGE2 secretion, indicating an anti-inflammatory response. Ovarian steroids and LH completely block LPS (10 ng/ml)-induced *TLR-4*, *IL-1 β* and *TNF- α* expression as well as LPS (100 ng/ml)-induced *TLR-2* expression. Taken together, this study suggests the existence of an early signaling system to respond to infection in the BOEC. In addition, ovarian steroids and LH may play a critical role in inducing homeostasis and in controlling hyperactive pro-inflammatory responses detrimental to epithelial cells, sperm and the embryo.

Key words: Bovine oviduct epithelial cell, Ovarian steroids, TLR-4, Th1/Th2 response

(J. Reprod. Dev. 59: 470–478, 2013)

The mammalian oviduct provides an optimal microenvironment for the activation and transportation of gametes, sperm capacitation, fertilization and early embryonic development, which is critical for the establishment of a successful pregnancy [1]. The oviduct is classically described as a sterile milieu even though pathogens and endotoxins can invade the mucosal surfaces of the oviduct *via* the uterus, peritoneal cavity and follicular fluid [2]. Therefore, the oviduct should be equipped with an efficient and strictly controlled immune system that would maintain optimal conditions for fertilization and early embryo development. Local immune responses, regulated by the secretions of epithelial cells, form a part of the mucosal innate immunity. In recent years, these responses, termed the epimmunome [3], have been recognized as critically important defense mechanisms, and their significance seems far greater than that of the systemic immune responses. The direct response of epithelial cells to microbial (i.e., pathogens) or nonmicrobial (i.e., stress or hormones) stimuli includes activation of the innate immune responses and regulation of subsequent adaptive immune responses [4]. This process is mediated by the secretion of different molecules

such as prostaglandins, chemokines and cytokines, which affect the conditioning of mucosal dendritic cells (DCs) [5]. Therefore, mucosal epithelial cells can orchestrate and provide early signals to drive immunity toward tolerance or inflammation.

The innate immune system recognizes pathogen-associated molecular patterns (PAMPs) using pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) [6, 7]. At least 10 bovine TLRs have been identified [8], and these PRRs bind to a range of microbial products and endogenous ligands. TLR signals are involved in the primary induction of inflammation as well as in the secondary activation of anti-inflammatory mechanisms [9]. For example, TLR-4 is a signal transducer of LPS, a component of the outer membrane of gram-negative bacteria. Ligation of TLR-4 by LPS leads to activation of nuclear factor kappa B (NF- κ B) and consequently transcription of pro-inflammatory cytokines and chemokines [10]. Another member of the TLR family, TLR-2, recognizes PAMPs associated with both gram-negative and gram-positive bacteria, including lipopeptides/lipoproteins, lipoteichoic acid, zymosan and components of peptidoglycan [11]. *E. coli* LPS enhances *TLR-2* mRNA and cell surface TLR-2 in the mouse [12], human [13, 14] and bovine [15] in an NF- κ B-dependent manner [16, 17]. Several investigations have shown the involvement of TLR-2 in LPS signaling [18, 19]. Engagement of TLR-2 in DCs by the bacterial lipopeptide Pam3Cys enhances extracellular signal-regulated kinase activation, resulting in stabilization of the transcription factor C-FOS, a suppressor of Th1 cytokines [20].

Received: April 4, 2013

Accepted: May 21, 2013

Published online in J-STAGE: June 21, 2013

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The oviduct is exposed to the female sex hormones, and these hormones clearly influence the immune system [21]. The bovine oviduct and BOEC express receptors for LH [22], E2 and P4 [23]. Estradiol and P4 have been shown to completely block *E. coli*-induced I κ B α phosphorylation and NF- κ B nuclear translocation in human cord blood mononuclear cells [24]. Sex hormones exert control over many chemokines/cytokines in the female reproductive tract. For example, P4 withdrawal results in increased expression of MCP-1 and IL-8, leading to chemotaxis and activation of monocytes and neutrophils, which results in release and activation of matrix metalloproteinases and initiation of menstruation [25].

It is clear that any changes in the balance between pro-inflammatory and anti-inflammatory responses (Th1 vs. Th2) during infection or a particular physiological situation can contribute to low defense against infection, resulting in increasing susceptibility to infection or low tolerance to pathogens. This, in turn, allows a cytotoxic immune response to be generated against antigens present on the normal epithelium cells, allogeneic sperm and a new semi-allogeneic embryo. It is therefore hypothesized that the BOEC can differentially respond to pathophysiological (i.e., LPS) and physiological (i.e., sex hormones) stimuli through the secretion of different cytokines and chemokines and the use of specific receptors, TLR-4 and TLR-2. We therefore investigated the regulation of local immune responses, Th1- or Th2- type responses, induced by *E. coli* LPS, ovarian steroids and LH at the levels around ovulation and their interactions in the BOEC *in vitro*.

Materials and Methods

Primary bovine oviduct epithelial cell (BOEC) isolation and culture

Epithelial cells were isolated as previously described [26, 27]. Briefly, fifteen oviducts were transported in an ice box from the local slaughterhouse to the laboratory, with the oviducts immersed in phosphate-buffered saline (PBS) solution without Ca²⁺/Mg²⁺ (PBS^{-/-}) (Sigma, St. Louis, MO, USA) but with 0.3% gentamicin (Sigma) and amphotericin B (Illkirch, Graffenstaden, France). They were cut and separated from the connective tissue, pathologically found to be healthy and washed twice with PBS. The lumen of oviducts was flushed with 15 ml PBS. BOECs were mechanically dislodged while being flushed with the same volume of PBS. Over a period of 15 min, the pooled sheets of BOECs from 2–3 cows settled at the bottom of the tube, and the cells were then washed with PBS followed by a medium consisting of D-MEM/F12, 0.1% gentamicin, 1% amphotericin and 2.2% NaHCO₃. Thereafter, the cells were harvested by centrifugation at 300 g for 10 min at 4 C. The resultant cell pellet was suspended in 10 ml PBS, layered over 10 ml Percol and centrifuged at 900 g for 20 min at 4 C. Finally, the cell pellet was washed once with the abovementioned medium, harvested by centrifugation at 300 g for 10 min at 4 C, and cultured in culture medium (D-MEM/F12, 0.1% gentamicin, 1% amphotericin and 2.2% NaHCO₃ supplemented with 10% fetal calf serum [FCS; BioWhittaker, Walkersville, MD, USA]) in 6-well culture dishes (Nalge Nunc International, DK-4000 Roskilde, Denmark) at 38.5 C in 5% CO₂ and 95% air. The following day, the BOEC culture was washed twice with PBS and incubated with culture medium supplemented with 5% FCS. After monolayer

formation, cells were trypsinized (0.05% trypsin EDTA; Amresco, Solon, OH, USA) until single cells appeared, and these cells were again plated in 6-well culture dishes at a density of 3×10^4 /ml and incubated at 38.5 C in 5% CO₂ and 95% air in culture medium supplemented with 5% FCS. The medium was renewed every 48 h until the growing BOEC monolayer covered 70–80% of the bottom of the culture plate. The purity of epithelial cell preparations was evaluated by reacting the cultured cells with monoclonal antibodies to cytokeratin (anti-cytokeratin-CK1) and immunostaining. The cells in the culture medium showed characteristic epithelial morphology. Approximately 98% of the cells were positive for anti-cytokeratin (CK1) antibodies.

LPS and hormone treatments

After the first passage but before the growing BOEC monolayer covered 70–80% of the bottom of the culture plate, in a preliminary study, the BOEC monolayer was washed twice with culture medium supplemented with 0.1% FCS and incubated for 24 h with 4 doses (1, 10, 100 and 1000 ng/ml) of LPS (serotype *E. coli* 055:B5; Sigma). In this dose-response study, 1000 ng/ml of LPS had lethal effects on cell viability, and 1 ng/ml of LPS showed a lack of significant responses (Fig. 2). Therefore, for further experimentation, doses of 10 and 100 ng/ml of LPS were used. BOECs were stimulated with one of the following: (1) LPS (10 or 100 ng/ml), (2) LPS (10 or 100 ng/ml) + LH (10 ng/ml, USDA-bLH-B6, USDA Animal Hormone Program, Bethesda, MD, USA), (3) LPS (10 or 100 ng/ml) + P4 (1 ng/ml, Sigma) or (4) LPS (10 or 100 ng/ml) + E2 (1 ng/ml, Sigma). As a control, culture medium without any LPS or hormones was added to the BOECs. The concentrations of hormones in this study were maintained similar to their physiological level in the bovine oviduct during the preovulatory period *in situ* [28]. This was done to mimic the local hormonal conditions around the time of ovulation in the oviduct when allogeneic sperm enters the oviduct and a semi-allogeneic embryo is about to start to develop. Finally, the medium was collected, and cells in the plates were trypsinized, washed twice with PBS^{-/-} and resuspended in 300 μ l PBS^{-/-}. A 10- μ l aliquot of the cell suspension was used to evaluate the cell viability. Cell viability was estimated using Trypan blue staining and was confirmed to be more than 90% at each time of plating as well as at the end of the experiment. The remaining cells were again separated by centrifugation at 300 g for 10 min at 4 C, lysed by TRIzol (Invitrogen, Carlsbad, USA) and stored at –80 C until RNA extraction.

Extraction of RNA, production of cDNA and real-time polymerase chain reaction (real-time PCR)

Total RNA was extracted from the BOECs using TRIzol (Invitrogen) as described in the protocol of Chomczynski and Sacchi [29]. The yield of extracted RNA for each sample was determined by ultraviolet (UV) spectroscopy (optical density, 260). The RNA concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany) at absorbances of 260 and 280 nm. The extracted total RNA was stored in RNA storage solution (Ambion, Austin, TX, USA) at –80 C until it was used for cDNA production. DNase treatment was carried out using an RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA) as described previously [30]. The synthesized cDNA was stored at –30 C. We analyzed the following genes: *TLR-4*, *TLR-2*,

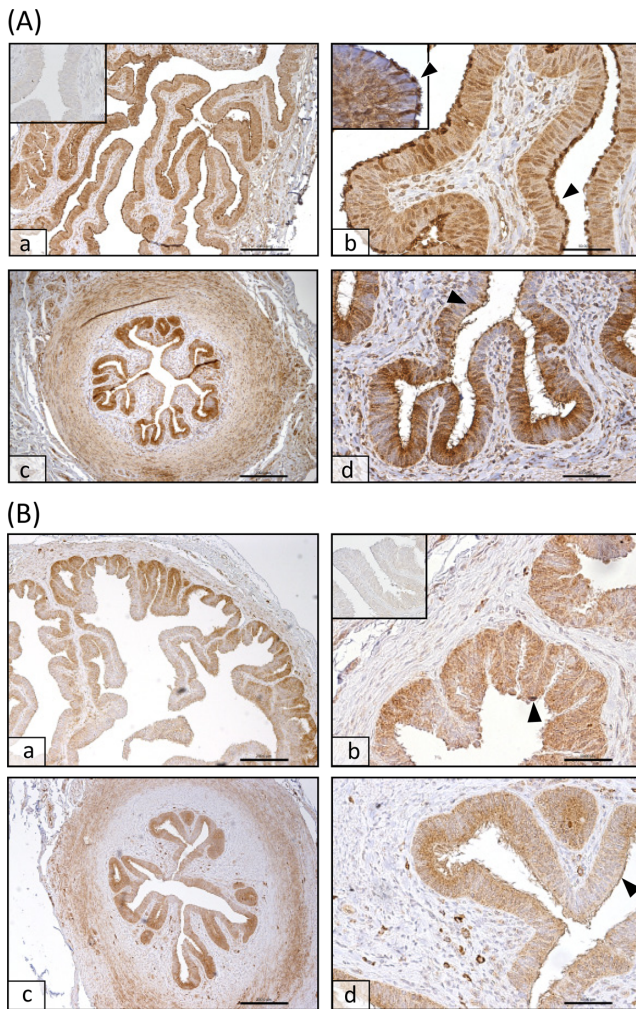


Fig. 1. (A) Immunohistochemical detection of TLR-2 in paraffin wax sections of the ampulla (a, b) and isthmus (c, d) of the bovine oviduct. The arrowhead (b, d) indicates the apical staining as well as staining of cells with apical cilia in the detailed picture in b. Insert in a: the control. Insert in b: detailed staining of cells with apical cilia. (B) Immunohistochemical detection of TLR-4 in paraffin wax sections of the ampulla (a, b) and isthmus (c, d) of the bovine oviduct. The arrowhead indicates the apical staining in d. Insert in b: the control. Scale bars represent 50 μm .

COX-2, *mPGES-1*, *TNF- α* , *IL1- β* , *IL-10*, *IL-4*, *NFKBIA*, *C-FOS* and *β -actin* (Table 1 lists primer sequences). Quantifications of mRNA expression were performed using synthesized cDNA *via* real-time PCR with a LightCycler (Roche Diagnostics, Mannheim, Germany) using a QuantiTect™ SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany). The primers were designed using Primer3 based on bovine sequences. The amplification program consisted of 15 min of activation at 95 C, followed by 40 cycles of PCR (15 sec of denaturation at 95 C, 30 sec of annealing at 54–58 C and 20 sec of extension at 72 C). The values of mRNA expression were assayed by normalization to β -actin as the internal standard. The expression of *β -actin* was stable in all experiments, and no significant difference

was detected in the levels of *β -actin* expression between treatments.

ELISA

The prostaglandin E2 measurements in the medium were performed without further preparation of medium, using a second antibody enzyme immunoassay (EIA) according to a previous study [28]. Briefly, the assay was performed using 96-well ELISA plates (Coming, NY, USA) coated with 100 μg of anti-rabbit IgG (Seikagaku, Tokyo, Japan). Basically, plates were incubated with 100 μl polyclonal antibody solution (1:600,000) for 24 h at 4 C. The following day, plates were decanted, and 15 μl of standards or samples were incubated with PGE2-HRP (1:40,000) for 24 h at 4 C.

Immunohistochemistry

The oviducts were transported from the local slaughterhouse to the laboratory while immersed in 0.9% saline solution in an ice box. The oviducts used in this study were from the preovulatory phase (days 19–20 of the cycle). The phase of the estrous cycle was identified as previously reported [28] based on the appearance of the corpus luteum, weight and color of the corpus luteum and follicular diameter. Paraffin-embedded tissue sections (4- μm thick) of the bovine ampulla or isthmus from the preovulatory phase were mounted on silane-treated glass slides (HistoBond Superior; Paul Marienfeld, Lauda-Königshofen, Germany) and dried at 37 C for 24 h. After drying, they were deparaffinized in xylene and rehydrated in a series of solutions of graded alcohol concentrations. To block endogenous peroxidase activity, the sections were incubated for 30 min in 80% alcohol solution containing 2% hydrogen peroxide. After rinsing the sections three times for 5 min in PBS (pH 7.2), antigen retrieval was performed by boiling the sections in 10 mM Tris Base and 1 mM EDTA solution (pH 9) for 30 min. The sections were then incubated for 20 min in 20% normal goat serum (in PBS) at room temperature to saturate any sites for nonspecific binding of proteins. The antibodies used for immunohistochemistry were rabbit anti-TLR-2 (1:100; orb11487, Biorbyt, Cambridge, UK) and rabbit anti-TLR-4 (1:200; 251111, Abbiotec, San Diego, CA, USA). The antibodies were diluted in PBS containing 1.5% bovine serum albumin and incubated in a humidifier overnight at 4 C. For detection, an EnVision™ anti-rabbit immunoglobulin conjugated to peroxidase-labeled dextran polymer system (DAKO, Glostrup, Denmark) was used in accordance with the manufacturer's protocol. Finally, sections were washed with PBS, and peroxidase activity was detected using DAB (Sigma, Steinheim, Germany) as a substrate for 5 min at room temperature. Sections were counterstained with hemalum, dehydrated, and mounted with DPX (Fluka, Buchs, Switzerland). To analyze nonspecific binding, primary antibodies were replaced with rabbit IgG (Sigma) at the same concentration as that used for the primary antibody.

Statistical analysis

Data are presented as the mean \pm SEM. Statistical analyses were performed with StatView 5.0 (SAS Institute). One-way ANOVA followed by multiple comparison tests, Fisher (3 groups) and Bonferroni (more than 3 groups), was performed, and all results were considered to be statistically significant at $P < 0.05$.

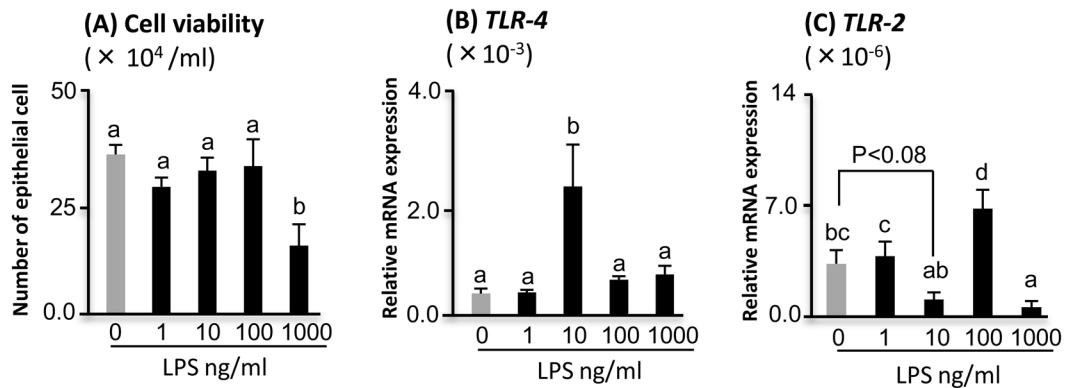


Fig. 2. Effect of O55:B5 *E. coli* lipopolysaccharide (0, 10, 100 and 1000 ng/ml) on (A) cell viability estimated with the use of trypan blue, (B) relative mRNA expression of *TLR-4* and (C) relative mRNA expression of *TLR-2* in a dose-dependent study in the bovine oviduct epithelial cell culture. Numerical values are presented as the mean ± SEM of 5 experiments. Different letters indicate significant differences between the treatments at $P < 0.05$ as determined by ANOVA followed by Bonferroni's multiple comparison test.

Table 1. Bovine primers were used in real-time PCR

Gene		Sequence of nucleotide (5'-3') *	Accession No.	Tm (C)	Product size (bp)
<i>TLR-4</i>	F	CTTGCGTACAGGTTGTTCTCTAA	NM_174198.6	56	153
	R	CTGGGAAGCTGGAGAAGTTATG			
<i>TLR-2</i>	F	GCTCCTGTGACTTCCTGTCC	NM_174197.2	54	501
	R	CCGAAAGCACAAGATGGTT			
<i>NFKBIA</i>	F	AAGTGGTCCGCCAAGTGAAG	NM_001045868.1	58	105
	R	CGATTTCTGGCTGGTTAGTGATC			
<i>C-FOS</i>	F	GAACGGAATAAGATGGCTGC	NM_001001162.1	58	220
	R	CCACAGACATCTCCTCTGGG			
<i>COX-2</i>	F	TCCTGAAACCCACTCCCAACA	AF031698	54	241
	R	TGGGCAGTCATCAGGCACAG			
<i>mPGES-1</i>	F	AGGACGCTCAGAGACATGGA	NM174443	58	142
	R	TTCGGTCCGAGGAAAGAGTA			
<i>IL-1β</i>	F	ATGAAAGACTGCATCCAACA	NM_174093.1	56	196
	R	ATGGAAGACATGTGCGTAGG			
<i>IL-4</i>	F	GCCACACGTGCTTGAACAAA	NM_173921.2	56	63
	R	TGCTTGCCAAGCTGTTGAGA			
<i>IL-10</i>	F	TTCTGCCCTGCGAAAACAA	NM_174088.1	58	85
	R	TCTCTGGAGCTCACTGAAGACTCT			
<i>TNF-α</i>	F	TGACGGGCTTTACCTCATCT	NM_173966.3	56	221
	R	TGATGGCAGACAGGATGTTG			
<i>β-actin</i>	F	CCAAGGCCAACCGTGAGAAAAT	K00622	58	256
	R	CCACATTCCGTGAGGATCTCA			

* F, forward; R, reverse.

Results

The bovine oviduct expresses proteins for TLR-4 and TLR-2

We investigated the expression of TLR-4 and TLR-2 in the bovine oviduct because it is hypothesized that the oviduct controls immune responses through variation in TLR signaling, which in turn, results in the production of different cytokines and chemokines. Figure 1 shows, by means of immunohistochemistry and intensive

staining of epithelial cells, the expression of TLR-4 and TLR-2 in the bovine oviduct.

Dose-dependent stimulation of BOECs by LPS results in different expressions of TLR-4 and TLR-2

A dose-response study was performed, in which epithelial cells were incubated in 0.1% FCS medium alone or in increasing doses of LPS (1, 10, 100 and 1000 ng/ml) for 24 h. LPS at a dose of

1000 ng/ml was toxic and significantly reduced the cell viability of BOECs (Fig. 2A, $P < 0.05$). The results also showed that BOECs are sensitive to LPS. Elevated expression of *TLR-4* indicated early-immune responses after administration of a low dose of LPS (10 ng/ml) (Fig. 2B, $P < 0.05$). Higher doses of LPS (100 and 1000 ng/ml) did not induce *TLR-4* expression. However, administration of 100 ng/ml LPS upregulated *TLR-2* expression while 1000 ng/ml LPS downregulated *TLR-2* expression (Fig. 2C, $P < 0.05$).

Immunological responses of BOECs to the different doses of LPS

Stimulation of BOECs with 10 ng/ml LPS significantly increased the expression of *TLR-4*, *NFKBIA*, *COX-2*, *IL1- β* and *TNF- α* (Fig. 3A–E, $P < 0.05$). However, 100 ng/ml LPS did not induce the expression of *TLR-4*, *NFKBIA*, *COX-2*, *IL1- β* and *TNF- α* but increased the expression of *TLR-2*, *IL-10* and *IL-4* (Fig. 3F, H and I, $P < 0.05$).

Ovarian steroids and LH completely block LPS-induced responses in the BOEC

The effect of ovarian steroids, E2 and P4, and LH on the pro-inflammatory response of the BOEC to LPS was investigated because the oviduct microenvironment is constantly exposed to sex hormones during the estrous cycle. BOECs were stimulated with LH (10 ng/ml) P4 (1 ng/ml) and E2 (1 ng/ml) at concentrations observed during the preovulatory period *in situ*. Ovarian steroids and LH completely suppressed LPS (10 ng/ml)-induced expression of *TLR-4*, *IL-1 β* and *TNF- α* (Fig. 4A, C and D, $P < 0.05$). Stimulation of BOECs with 10 ng/ml of LPS together with either P4 or LH also suppressed the stimulatory effect of LPS (10 ng/ml) on *COX-2* expression (Fig. 4E, $P < 0.05$). Stimulation of the cells by 10 ng/ml LPS together with either P4 or E2 reduced LPS (10 ng/ml)-induced *NFKBIA* expression (Fig. 4B, $P < 0.05$). Ovarian steroids and LH also inhibited the stimulatory effect of 100 ng/ml LPS on *TLR-2* expression (Fig. 4F, $P < 0.05$). Moreover, E2 inhibited stimulatory effects of 100 ng/ml LPS on *IL-10* expression (Fig. 4H, $P < 0.05$). Stimulation of BOECs with 100 ng/ml LPS together with E2 induced *C-FOS* expression compared with LPS (100 ng/ml)-stimulated cells (Fig. 4G, $P < 0.05$).

Regulation of prostaglandin E2 secretion by LPS, E2 and LH in the BOEC

A dose of 100 ng/ml of LPS significantly stimulated *mPGES-1* expression and PGE2 secretion in BOEC culture (Fig. 5A–B, $P < 0.05$).

Discussion

The results of this study provide evidence that the bovine oviduct expresses genes and proteins for both *TLR-4* and *TLR-2*. Intensive expression of these TLRs was found within the epithelial cells. Interestingly, *TLR-2* and *TLR-4* were highly expressed in the apical cilia rather than basolateral pole of the bovine oviduct epithelial cells. This suggests higher sensitivity of the oviduct epithelial cells to the possible TLRs ligands in the oviduct fluid. Previous studies showed that *TLR-2*, but not *TLR-4*, is expressed in the murine oviduct [31]. The human oviduct has been shown to express *TLR-2*, and *TLR-4* is expressed within oviductal stromal fibroblasts but not oviductal epithelial cells [32, 33]. These findings indicate the existence of a

species-specific difference in oviduct mucosal immunity.

In this study, 10 ng/ml LPS induced expression of *TLR-4* but not *TLR-2*, and a 10-fold higher concentration of LPS increased the expression of *TLR-2* but not *TLR-4*. Heath *et al.* [34] reported that 1000 ng/ml *E. coli* LPS induced *TLR-4* expression in the endometrial cells of the bovine uterus, and Ibeagha-Awemu *et al.* [15] found that 0.01–10 μ g/ml LPS stimulates the expression of both *TLR-4* and *TLR-2* in bovine mammary epithelial cells. The absence of an effect of LPS at the dose of 100 ng/ml on the expression of *TLR-4* could be due to the concomitant upregulation of Th2 cytokines such as *IL-4*. It has been shown that *IL-4* reduces LPS responsiveness and *TLR-4* protein surface expression, *TLR-4* mRNA expression and transcriptional activity of the upstream region of *TLR-4* in intestinal epithelial cells and PBMCs [35, 36]. This effect is dependent on tyrosine kinase and STAT6. A STAT6 binding site has been determined in an area of the *TLR-4* gene necessary to mediate the inhibitory effects of *IL-4* on *TLR-4* transcription [36]. The present data may imply a novel autonomous innate immunity in the bovine oviduct.

Exposure of epithelial cells to 10 ng/ml LPS induced the expression of *TLR-4*, *COX-2*, and Th1 cytokines (i.e., *IL-1 β* and *TNF- α*), demonstrating a high sensitivity of oviduct epithelial cells to low-dose LPS. Interleukin-1 β and *TNF- α* are known to be involved in the inflammatory responses of the human oviduct to *Chlamydia trachomatis* infection [37, 38]. The induction of *COX-2*, an important downstream target of *TLR-4* signaling [39], increases epithelial production of PGE2 and causes proliferation in infected epithelial cells [40]. The present results imply that the bovine oviduct epithelial cell may employ an early and sensitive innate surveillance (manifested by elevated *TLR-4* expression) to preclude infection. This could be sufficient to provide a sterile microenvironment and, at the same time, protection from infection.

Interestingly, low-dose LPS also stimulated the expression of *NFKBIA*, which is NF- κ B-dependent. *NFKBIA* strongly sequesters NF- κ B in the cytoplasm, thereby generating autoregulatory feedback loops in the NF- κ B response [41]. Rodrigues *et al.* [42] found increased *NFKBIA* expression in the rat epididymis challenged by *E. coli* LPS after 2 h of exposure *in vivo*. Thus, in the present study, a low dose of LPS induced *NFKBIA* expression, possibly providing a compensatory mechanism to balance activation of NF- κ B.

Importantly, 100 ng/ml LPS did not induce *TLR-4* and Th1 cytokines but stimulated the expression of *TLR-2* and Th2 cytokines (i.e., *IL-4* and *IL-10*). We propose two possibilities to explain this phenomenon (Fig. 6). First, the high levels of *IL-1 β* and *TNF- α* during infection are clearly detrimental to the human oviduct [37, 38], leading to barrier disruption, subsequent translocation of bacteria to the lamina propria [43] and disturbance of the natural tolerance mediated by regulatory T cells and immunosuppressive cytokines [44]. Because of this, it is hypothesized that the BOEC responds to 100 ng/ml LPS through suppression of *TLR-4* and Th1 cytokines to prevent severe tubal injury. Moreover, *TLR-2* critically participates in protection of the intestinal epithelial barrier and controls mucosal inflammation by directly preserving tight junctions [11]. Thus, upregulation of *TLR-2* by 100 ng/ml LPS may also involve in preserving oviductal epithelial barrier. In addition, according to the theory of Th2-mediated repair, many of the proteins produced in response to *IL-4* are associated with injury and have well-known roles in tissue repair [45].

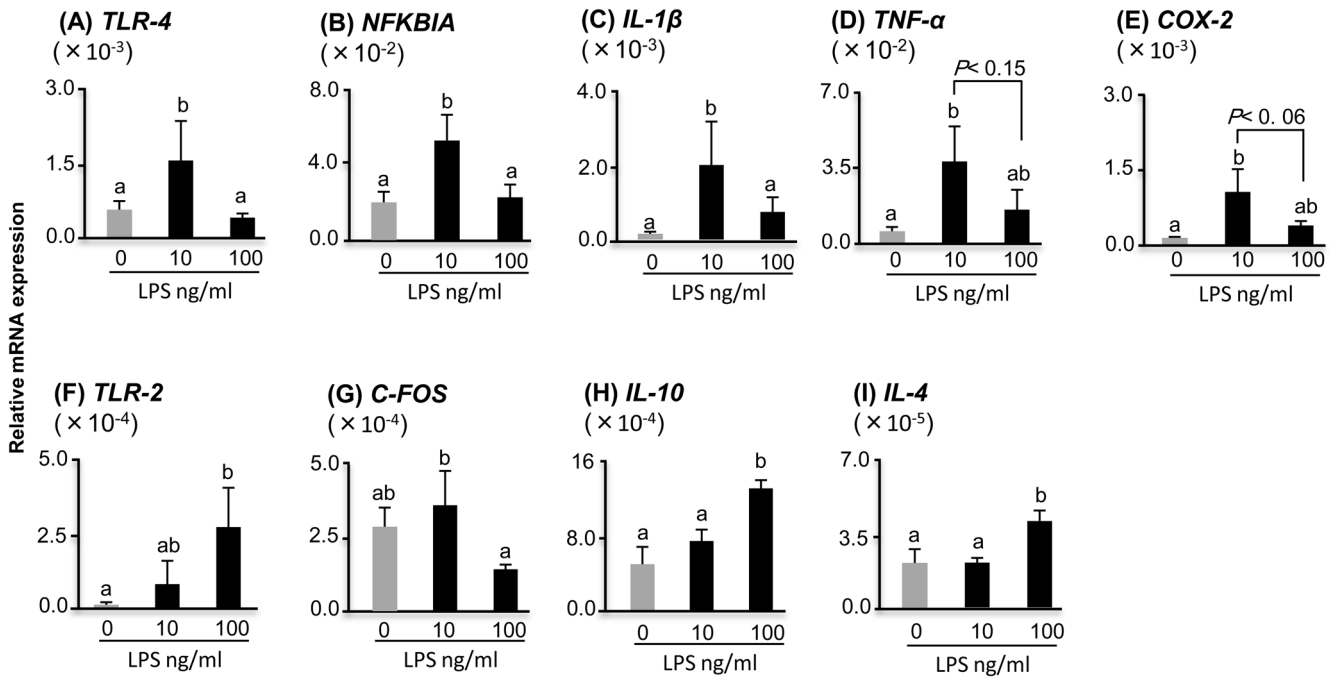


Fig. 3. Relative mRNA expression of *TLR-4*, *COX-2*, *IL-1 β* , *TNF- α* , *TLR-2*, *C-FOS*, *IL-10*, *IL-4* and *NFKBIA* in the bovine oviduct epithelial cell culture stimulated with different doses of O55:B5 *E. coli* lipopolysaccharide (10 and 100 ng/ml) and harvested after 24 h. Numerical values are presented as the mean \pm SEM of 5 experiments. Different letters indicate significant differences between the treatments at $P < 0.05$ as determined by ANOVA followed by Fisher's multiple comparison test.

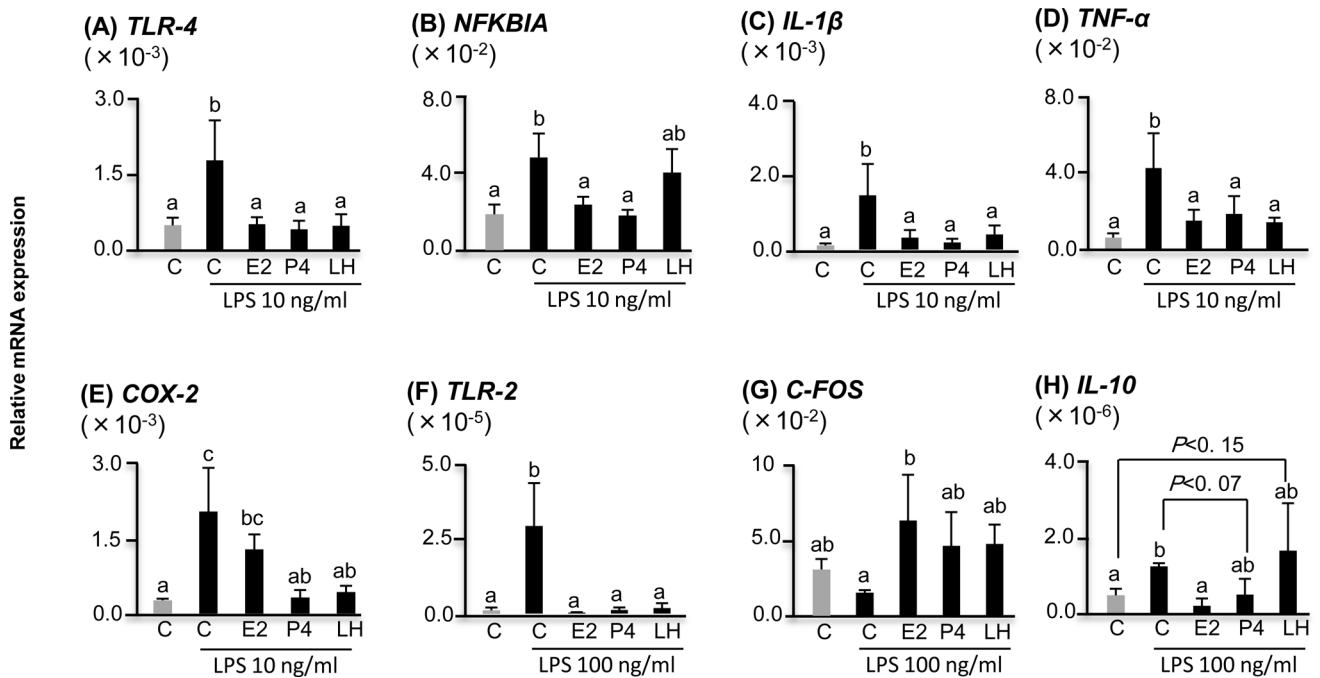


Fig. 4. Relative mRNA expression of *TLR-4*, *COX-2*, *IL-1 β* , *TNF- α* , *TLR-2*, *C-FOS*, *IL-10*, *IL-4* and *NFKBIA* in the bovine oviduct epithelial cell culture stimulated with different doses of O55:B5 *E. coli* lipopolysaccharide (10 and 100 ng/ml) together with E2 (1 ng/ml), P4 (1 ng/ml) and LH (10 ng/ml) at concentrations observed during the preovulatory period *in situ* and harvested after 24 h. C (gray bar): control without any stimulant. Numerical values are presented as the mean \pm SEM of 5 experiments. Different letters indicate significant differences between the treatments at $P < 0.05$ as determined by ANOVA followed by Bonferroni's multiple comparison test.

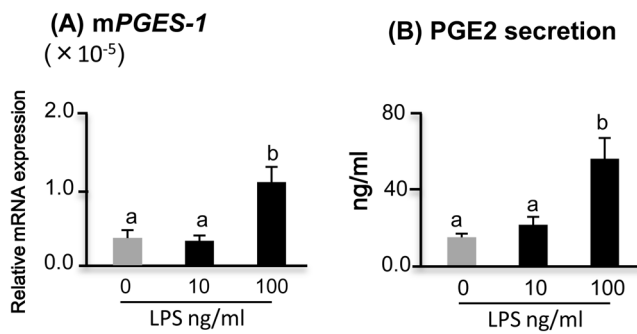


Fig. 5. Effect of different doses of O55:B5 *E. coli* lipopolysaccharide (0, 10 and 100 ng/ml) on (A) mRNA expression of *mPGES-1* and (B) PGE2 secretion in the bovine oviduct epithelial cell culture. C (gray bar): control without any stimulant. Numerical values are presented as the mean \pm SEM of 5 experiments. Different letters indicate significant differences between the treatments at $P < 0.05$ as determined by ANOVA followed by Fisher's multiple comparison test.

Second, although TLRs are crucial for an efficient immune response, certain pathogens use TLR-based strategies to evade the host defense, preventing further elimination of the microorganisms [9]. TLR-2, which is a strong mediator of anti-inflammatory effects [9], and TLR-4 are involved in *IL-10* expression [46]. *E. coli* LPS induces *TLR-2* expression and sensitivity of TLR-2 to stimulations [12–14]. LPS is known to signal through TLR-2 [47]. *E. coli* LPS *in vitro* activates spleen cells from TLR-2^{+/+} mice to induce production of the Th2-polarizing cytokine IL-10 [48]. Moreover, in the presence of myeloid differentiation protein 2 (MD-2, a protein associated with TLR-4 on the cell surface and needed for TLR-4 to respond to LPS), both TLR-2 and TLR-4 are highly and equally sensitive to all endotoxic LPS, including protein-free LPS [49]. MD-2 enhances TLR-2-mediated responsiveness to both gram-negative and gram-positive bacteria [49]. The present results suggest that a dose of 100 ng/ml of LPS increases the expression of TLR-2, which is accompanied by elevated expression of Th2 cytokines, and the concomitant decrease in the expressions of *TLR-4* and Th1 cytokines. It is proposed that this arrangement is related to mucosal homeostasis *via* mucosal barrier protection and the prevention of expanding inflammation. It may also serve as a mechanism of escape from the oviduct defense for gram-negative bacteria.

A dose of 100 ng/ml LPS stimulated the expression of *mPGES-1* and PGE2 secretion but not *COX-2* expression. It is documented that both TLR-4 and TLR-2 are involved in PGE2 secretion [50, 51]. LPS stimulates PGE2 production in bovine endometrial cells [34]. TLR-2 is also the major receptor that mediates PGE2 production in response to *C. albicans* in murine peritoneal macrophages and splenocytes [51]. The present results suggest that elevated expression of *TLR-2* in cells treated with 100 ng/ml could induce *mPGES-1* expression and PGE2 secretion. Interestingly, despite the well-known regulatory coupling of the two enzymes, *COX-2* and *mPGES-1*, LPS at a dose of 10 ng/ml induced *COX-2* expression but not *mPGES-1* expression and resultant PGE2 secretion. Arosh *et al.* [52] also reported that *mPGES-1* expression is not coupled to the expression of *COX-2* in

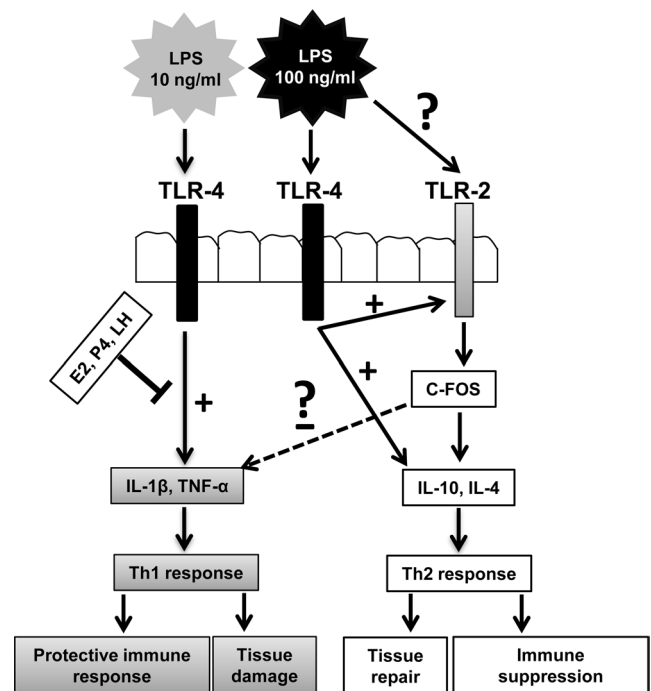


Fig. 6. Schematic illustration for a novel regulation of local innate immunity in the bovine oviduct. Differential TLR-mediated pathways can induce specificity to innate immunity. Recent literature suggests that certain microorganisms not only are recognized by TLRs for the activation of host defense but also activate alternative TLR pathways with inhibitory effects on innate immunity. Interaction of 10 ng/ml *E. coli* LPS with TLR4 induces expression of Th1 cytokines, which switches to a protective immune response, while activation of TLR-4, together with possible TLR-2 signaling by 100 ng/ml *E. coli* LPS, induces the expression of Th2 cytokines, which switches to an immune suppression. This results in inhibition of host defense and increased susceptibility to infection. Similar mechanisms have been suggested for other microorganisms, such as *Y. enterocolitica* and *A. fumigates* [20]. On the other hand, Th2 cytokines and TLR-2 play important roles in preserving tight junctions and tissue repair. Moreover, ovarian steroids and LH markedly influence innate immune protection by epithelial cells in the oviduct through inhibition of pro-inflammatory responses.

the endometrium stimulated with interferon-tau. It seems that the expression of *COX-2* and *mPGES-1* in response to LPS may also be uncoupled in the BOEC. Therefore, PGE2 secretion could be controlled by *mPGES-1* but not *COX-2* in BOEC culture in response to LPS.

An important finding of our study is that E2, P4 and LH, at the concentrations observed around ovulation *in situ*, completely reversed the stimulatory effect of 10 ng/ml LPS on *TLR-4* expression and TLR4-related genes. Moreover, E2, P4 and LH suppressed *TLR-2* expression in response to 100 ng/ml LPS, and E2 reversed LPS induction of *IL-10* expression. Fahey *et al.* [53] reported that E2 can reverse the stimulatory effects of IL-1 β on the expression of *TNF- α* , *IL-8* and *NF- κ B* in human uterine epithelial cells. It has been shown that the inhibitory effects of E2 and P4 on LPS-induced immune responses are receptor mediated in human uterine epithelial cells and murine bone marrow stem cells [53, 54]. Since the bovine oviduct and

BOEC express receptors for LH [22], E2 and P4 [23], the inhibition of LPS-induced TLR-4 and TLR-4 related genes by these hormones may be mediated through receptors in the BOEC. The present data imply that the periovulatory levels of E2, P4 and LH accomplish downregulation of both Th1 and Th2 responses during infection to prevent harmful changes in the oviduct microenvironment, particularly when allogeneic sperm enters the oviduct and a semi-allogeneic embryo is about to start to develop. However, this may lead to loss of immune responses, resulting in tissue damage by bacteria or toxins. It has been reported that despite suppression of LPS-induced NF- κ B and proinflammatory responses by E2 in human uterine epithelial cells, E2 induces antimicrobial protein secretory leukocyte protease inhibitor (SLPI) [53]. Therefore, on the one hand, these hormones may enhance protection against pathogens by increasing antimicrobial activity. On the other, they may ensure an environment for successful fertilization by suppressing proinflammatory responses detrimental to allogeneic sperm and a semi-allogeneic embryo. However, if the invasion of infection overwhelms the immune capacity, the affected cells or tissue appear to be endangered by bacteria or toxins.

Taken together, these findings shed a light on a novel immune function of the BOEC, which initiates a biphasic and sensitive proinflammatory response to infection. A low dose of *E. coli* LPS initiate a Th1-type response, but at a higher LPS dose, the immune response switches to a Th2-type response. In addition, ovarian steroids and LH suppress LPS-induced pro-inflammatory responses. Therefore, epithelial cells employ ovarian steroids and LH in maintaining microenvironmental homeostasis of the oviduct, guaranteeing an optimal microenvironment to achieve successful fertilization *via* inhibition of Th1 responses detrimental to allogeneic sperm and the semi-allogeneic embryo in the bovine oviduct. Since the responses to LPS and hormones could be modulated by other cells and factors as well as epithelial cells in the oviduct, further investigation will focus on the possible interactions among epithelial cells, immune cells and other cell types.

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