Pathogen-Associated Molecular Patterns Initiate Inflammation and Perturb the Endocrine Function of Bovine Granulosa Cells From Ovarian Dominant Follicles via TLR2 and TLR4 Pathways

Jennifer C. Price, John J. Bromfield, and I. Martin Sheldon

Institute of Life Science, School of Medicine, Swansea University, Singleton Park, Swansea SA2 8PP, United Kingdom

Bacterial infections of the uterus or mammary gland commonly cause disease and infertility by perturbing growth and steroidogenesis of the dominant follicle in the ovary of cattle. Cells of the innate immune system use Toll-like receptors TLR2, TLR4, and TLR5 to recognize pathogen-associated molecular patterns (PAMPs) expressed by bacteria, leading to activation of MAPK and nuclear factor-κBκ pathways and production of inflammatory cytokines such as IL-1β and IL-6, and the chemokine IL-8. The present study tested whether granulosa cells from dominant follicles have functional TLR2, TLR4, and TLR5 pathways. Supernatants of primary bovine granulosa cells accumulated IL-1β, IL-6, and IL-8 when treated for 24 hours with Pam3CSK4 (PAM) that binds TLR2 or lipopolysaccharide (LPS) that binds TLR4 but not flagellin that binds TLR5. Granulosa cell responses to PAM or LPS were rapid, with increased phosphorylation of p38 and ERK1/2 within 30 minutes and increased abundance of *IL6, IL1B, IL10, TNF, IL8*, and *CCL5* mRNA after 3 hours of treatment. Accumulation of IL-6 in response to PAM and LPS was attenuated using small interfering RNA targeting *TLR2* and *TLR4*, respectively. Furthermore, treating granulosa cells with inhibitors targeting MAPK or nuclear factor-κB reduced the accumulation of IL-6 in response to LPS or PAM. Treatment with LPS or PAM reduced the accumulation of estradiol and progesterone, and the PAMPs reduced granulosa cell expression of CYP19A1 mRNA and protein. In conclusion, bacterial PAMPs initiate inflammation and perturb the endocrine function of bovine granulosa cells from dominant follicles via TLR2 and TLR4 pathways. **(*Endocrinology* 154: 3377–3386, 2013)**

Infections of the uterus or mammary gland with bacteria commonly cause disease in dairy cattle, and these infections are associated with infertility (1–4). After parturition, ~90% of animals have bacterial infections of the uterus and 40% develop clinical disease, which costs the US dairy industry $600 million every year, mainly because of reduced fecundity (2). Bacterial infection of the mammary gland causes mastitis in a similar proportion of animals, and these infections also reduce fecundity (5). Animals with mastitis have reduced follicle growth rates, altered granulosa cell gene expression, and lower dominant follicle estradiol concentrations (3). Similarly, uterine disease not only damages the endometrium but also affects ovarian function, with slower growth of the dominant follicle in the ovary, perturbed ovulation, and reduced steroidogenesis (1, 2). The conceptual question is how bacterial pathogens distant to the ovary might affect the dominant follicle. Potential explanations include the impact of circulating inflammatory mediators, changes in metabolites during infection, or bacteria ascending the oviduct from the uterus. However, the simplest rationale is a direct effect

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Abbreviations: DMSO, dimethyl sulfoxide; D-PBS, Dulbecco's phosphate-buffered saline; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FACS, fluorescence-activated cell sorting; FLA, flagellin; FSHR, FSH receptor; JNK, c-Jun N-terminal kinase; LHCGR, luteinizing hormone receptor; MEK, mitogen-activated protein kinase kinase; MHC, major histocompatability complex; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NFκB, nuclear factor-κB; PAM, Pam3CSK4; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; siRNA, small interfering RNA; TLR, Toll-like receptor.

of bacterial molecules on the dominant follicle in the ovary.

In cattle, a single dominant follicle is selected from a cohort of smaller follicles, which develop in waves in response to increased concentrations of FSH in peripheral plasma (6). The characteristics of a dominant follicle include a diameter of >8.0 mm, increased expression of *CYP19A1* (also called aromatase), increased conversion of androstenedione to estradiol, and expression of luteinizing hormone receptors (LHCGRs) on granulosa cells, in addition to their existent FSH receptors (FSHRs) (6). Granulosa cells regulate dominant follicle growth and function and are involved in ovulation in response to LH induction of epidermal growth factors (EGFs), which bind EGF receptors (EGFRs) on granulosa cells to induce a cascade of events resembling inflammation (7, 8). However, it is not known whether there are mechanisms linking inflammation caused by bacterial infections with physiological inflammation of granulosa cells associated with ovarian function. The bacteria that infect the uterus or mammary gland of cattle include Gram-negative organisms, particularly *Escherichia coli*, and Gram-positive bacteria such as *Trueperella pyogenes* and *Staphylococcus aureus* (1, 2, 4, 5). Although little is known about Gram-positive bacteria, the lipopolysaccharide (LPS, endotoxin) cell wall component of Gram-negative bacteria accumulates in the follicular fluid of dominant follicles in vivo, and LPS perturbs estradiol production by granulosa cells collected from dominant follicles (9). It is important to test whether granulosa cells have roles in immunity because there are no immune cells within the basement membrane of healthy ovarian follicles (10, 11).

The Toll-like receptor (TLRs) family of 10 cellular receptors is responsible for detecting and initiating the innate immune defense against microbes by binding their pathogen-associated molecular patterns (PAMPs) (12, 13). The prototypical PAMP from Gram-negative bacteria is LPS, which binds TLR4 in complex with coreceptors cluster of differentiation 14 and myeloid differentiation factor-2. Lipoproteins from Gram-positive or Gram-negative bacteria are bound by TLR2, although issues of purifying native PAMPs mean that most in vitro work uses the synthetic triacylated lipoprotein Pam3CSK4 (PAM) as the ligand for TLR2 (12, 13). Finally, TLR5 binds flagellin (FLA) from motile bacteria that possess flagella. Activation of TLR signaling typically results in phosphorylation of MAPK cascades, particularly ERK1/2, c-Jun N-terminal kinase (JNK), and p38, and nuclear translocation of nuclear factor-κB (NFκB) components, which leads to production of proinflammatory cytokines, typically IL-1β, IL-6, and TNFα, and chemokines such as IL-8 (12, 13). Although TLRs are primarily associated with cells of the innate immune system, such as macrophages and neutrophils, bovine and murine granulosa cells express mRNA for the TLR4 complex (9, 14). Furthermore, bovine granulosa cells collected from ovarian antral follicles before dominance generate an inflammatory response to LPS via TLR4 (10). However, it is important to determine whether granulosa cells from dominant follicles can mount an inflammatory response to bacterial PAMPs because bacterial infections in vivo principally affect dominant follicle growth and function (2, 3).

In the present study, we tested the hypothesis that granulosa cells from dominant follicles have functional TLR2, TLR4, and TLR5 pathways. The inflammatory response and activation of intracellular pathways were investigated using pure populations of primary bovine granulosa cells treated with PAM, LPS, or FLA. Primary granulosa cells collected from dominant follicles expressed mRNA for all 10 TLRs, and PAM and LPS increased cytokine and chemokine abundance via the TLR2 and TLR4 pathways, respectively, although there was no detected response to the TLR5 ligand FLA. The granulosa cell responses to PAM or LPS were associated with increased phosphorylation of p38 and ERK, and inhibitors targeting MAPKs or NFκB reduced the cellular response to these PAMPs. Treatment with LPS or PAM also reduced the accumulation of estradiol and progesterone and the expression of CYP19A1, which is important for granulosa cell endocrine function. The inflammatory responses to PAMPs via the TLR2 and TLR4 pathways in granulosa cells provide a molecular explanation of how bacterial infections outside the ovary may perturb ovarian dominant follicle function.

Materials and Methods

Cell isolation and culture

Ovaries were collected from 2.2 ± 0.1-year-old mixed-breed beef cattle within 15 minutes of slaughter and transported to the laboratory on ice in Dulbecco's phosphate-buffered saline (D-PBS) (Sigma-Aldrich, Gillingham, United Kingdom) containing 10% penicillin/streptomycin (Sigma-Aldrich). All animals were healthy with no gross evidence of infection of the uterus or mammary gland to avoid the confounding effects of existent PAMPs. Ovaries from between 6 and 10 animals were pooled for each experiment, with approximately 340 animals used across all experiments. Within 90 minutes of excision, ovaries were processed for collection of mural granulosa cells. Ovaries were rinsed in 70% ethanol followed by a brief rinse in sterile PBS (Sigma-Aldrich). An LPS-free 2-mL syringe and a 20-gauge needle were used to aspirate dominant follicles, as determined by an external diameter of >8.0 mm (15), into collection medium comprising Medium 199 (Invitrogen, Paisley, United Kingdom) supplemented with 0.25% (w/v) BSA, 25 mM HEPES, 0.005% (w/v)

heparin, and 1% penicillin/streptomycin (all from Sigma). Granulosa cells were pooled and washed twice in granulosa cell culture medium comprising Medium 199 supplemented with 10% fetal calf serum (Biosera, Ringmer, United Kingdom), 1% penicillin/streptomycin, 1% L-glutamine, and 1% insulin, transferrin, and sodium selenite liquid media supplement (all from Sigma-Aldrich). Granulosa cells were then resuspended and plated at 1.5×10^6 cells/mL in 1 mL, 0.5 mL, or 100 μL of culture medium in 12-, 24-, or 96-well plates (TPP, Trasadingen, United Kingdom), respectively. For analysis by semiquantitative RT-PCR or Western blot, 12-well plates were used; for analysis by ELISA, 24-well plates were used; and to test inhibitors, 96-well plates were used. Each experiment was performed at least 3 times, with each treatment in single (12- and 24-well plates) or duplicate (96-well plates) wells.

Blood was collected into heparinized tubes from the jugular vein within 1 minute of killing and transported to the laboratory at room temperature. Blood was pelleted at $700 \times g$ for 10 minutes, and the buffy layer was removed using a sterile Pasteur pipette into a sterile 15-mL centrifuge tube. The buffy coat was washed using D-PBS before lysing of residual red blood cells using sterile water; the resultant peripheral blood mononuclear cells (PBMCs) were washed again, resuspended at 1×10^6 cells/mL in D-PBS, and used as a positive control for flow cytometry and RT-PCR.

Evaluation of granulosa cell responses to PAMPs

To evaluate granulosa cell responses to PAMPs, cells were cultured for 48 hours, washed with sterile D-PBS, and treated with control culture medium or medium containing ultrapure LPS from *Escherichia coli* O111:B4 (10-fold increasing range of concentrations from 0.001 to 10 μg/mL; Invivogen, Toulouse, France), PAM (10-fold increasing range of concentrations from 0.001 to 10 μg/mL; InvivoGen), or FLA from *Salmonella typhimurium* (10-fold increasing range of concentrations from 0.0001 to 1 μg/mL; InvivoGen); the potencies of LPS, PAM, and FLA were previously confirmed by IL-6 and IL-8 accumulation from bovine endometrium (16). In subsequent experiments, the granulosa cells were treated with medium containing 1 μg/mL LPS or 1 μg/mL PAM for 5 to 25 minutes for analysis by Western blot, for 3 hours to examine mRNA expression or for 24 hours before collection of supernatants for ELISA analysis. When the accumulation of steroid hormones was elevated, the cells were cultured in the presence of 1 ng/mL highly purified bovine FSH (A. F. Parlow, National Hormone and Peptide Program, Torrance, California) and 10^{-7} M androstenedione (Sigma-Aldrich) (9). To explore the role of TLRs, granulosa cells were transfected with Lipofectamine RNA iMax (Invitrogen) containing 10 pmol of small interfering RNA (siRNA) targeting TLR2 (sense, GGACAGAAUUAGACACCUAUU; antisense, UAGGUGUCUAAUUCUGUCCUU) or TLR4 (sense, GAGUAUAUCUUUAGGAAGUUU; antisense, ACUUCCUAAAGAUAUACUCUU), or scramble siRNA (ON-TARGETplus Non-targeting siRNA #1; all siRNA was from Thermo Scientific, Cramlington, United Kingdom) and cultured in antibiotic-free culture medium for 24 hours. The cells were then washed with D-PBS and lysed using buffer RLT for analysis of TLR mRNA expression or treated with control culture medium or medium containing 1 μg/mL LPS or PAM. After 24 hours, supernatants were collected and stored at −20°C for analysis by ELISA, and cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (see below).

Inhibitors

To explore the role of intracellular signaling pathways, granulosa cells seeded in 96-well plates were cultured for 48 hours, washed with D-PBS, and then treated for 30 minutes with 50 μL of medium containing dimethyl sulfoxide (DMSO) (1:500; Sigma-Aldrich) or medium containing inhibitors, before addition of 50 μL of control medium or medium containing LPS or PAM to give a final concentration of 1 μg/mL. The inhibitors were dissolved in DMSO (maximal concentration of 1:500), and treatments spanned 10-fold increasing concentrations of inhibitors targeting NFκB (NFκB activation inhibitor InSolution, final concentration ranging from 0.0004 to 40 μM; Calbiochem, San Diego, California), JNK (JNK inhibitor II, 0.005–500 μM; Calbiochem), p38 (SB230580, 0.001–100 μM; Calbiochem), or mitogen-activated protein kinase kinase (MEK) (U0126, 0.001–10 μM; Calbiochem). After 24 hours, supernatants were collected and stored at −20°C for analysis by ELISA, and cell viability was assessed by the MTT assay (see below).

ELISA

The accumulation of IL-6, IL-1β, TNFα, IL-8, estradiol, and progesterone in cell-free supernatants was measured in duplicate using ELISA, according to the manufacturers' instructions (bovine IL-6 and bovine IL-1β, Thermo Scientific; bovine TNFα and human IL-8, which has established bovine cross-reactivity [10], R&D Systems, Abingdon, United Kingdom; estradiol and progesterone, DRG Diagnostics, Marburg, Germany). The interassay and intra-assay coefficients of variation were all <8% and 5%, respectively. The limits of detection were 35.6 pg/mL for IL-6, 20.1 pg/mL for IL-1β, 21.8 pg/mL for TNFα, 14.3 pg/mL for IL-8, 10.7 pg/mL for estradiol, and 0.04 ng/mL for progesterone.

MTT assay

To examine cell viability at the end of experiments, supernatants were removed, and the cells were incubated with fresh medium containing 0.5 mg/mL MTT (Sigma-Aldrich) for 1 hour (17). The medium was removed, and the cells were washed with D-PBS before lysis with DMSO and measurement of absorbance at 570 nm using a microplate reader (POLARstar Omega; BMG Labtech, Offenburg, Germany).

RNA extraction and semiquantitative RT-PCR

Total RNA was isolated from PBMCs and granulosa cell samples after 2 washes in PBS. Samples were resuspended in RLT buffer (QIAGEN, Crawley, United Kingdom) before being passed through a ribonuclease-free 20-gauge needle 10 times to disrupt cells. Total mRNA extraction was performed using the RNA Easy Mini kit (QIAGEN) according to the manufacturer's instructions. Total mRNA was measured using a spectrophotometer (ND 2.0; NanoDrop Technologies, Wilmington, Delaware) and reverse-transcribed according to the manufacturer's instructions using the QuantiTect Reverse Transcription Kit (QIAGEN) and 1 μg of RNA/reaction.

Primers were designed for genes encoding TLR1–TLR10, inflammatory mediators (*IL6, IL1B, TNF, IL10, IL8*, and *CXCL5*), *ACTB, AMH, MHCII, CD45, FSHR, LHCGR,*

EGFR, and *CYP19A1*, using the National Center for Biotechnology Information database, and specificity was determined using Primer-BLAST (please see Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Real-time PCR was performed with an iQ5 light cycler (Bio-Rad, Hemel Hempstead, United Kingdom) using QuantiFast SYBR Green (QIAGEN) in 25-μl reactions containing 1 μM concentrations of each forward and reverse primer (Sigma Genosys, Haverhill, Suffolk, United Kingdom). The PCR conditions were activation at 95°C for 5 minutes, 40 cycles of denaturation at 95°C for 10 seconds, and annealing/extension at 60°C for 30 seconds; a melt curve was generated to estimate specificity. Reference cDNA was used to generate a standard curve to determine the starting quantity of mRNA in each sample, with expression of each gene normalized to the reference gene *ACTB*. In the present study, the efficiency of all primers was 100 ± 10%, and there was no significant effect of LPS or PAM treatment on *ACTB* expression (fold change compared with control: LPS, 1.00 ± 0.09; PAM, 1.09 ± 0.08).

Western blotting

Cells were cultured for 24 hours in a 12-well plate, washed twice with D-PBS, and then cultured in Opti-MEM medium (Invitrogen) for 24 hours. They were then treated for 0, 5, 10, 15, 20, or 25 minutes or for 24 hours with Opti-MEM medium or Opti-MEM medium containing 1 μg/mL LPS or PAM. The medium was then discarded before the cells were washed with D-PBS and 80 μl of PhosphoSafe Extraction Reagent (Novagen, Darmstadt, Germany) used for cell lysis. Protein was extracted and quantified using the DC assay (Bio-Rad), and 10 μg of protein was electrophoresed on a 12% PROTEAN TGX gel (Bio-Rad) for 45 minutes at 200 V. Protein was transferred onto a polyvinylidene difluoride membrane (GE Healthcare, Chalfont St Giles, United Kingdom) for 90 minutes at 400 mA before blocking overnight at 4°C in 5% BSA in Tris-buffered saline–Tween 20. Blots were probed using antibodies diluted 1:1000 in block solution for diphosphorylated ERK1/2 (8159; Sigma-Aldrich), phosphorylated p38 (APO5898PU-N; Acris Antibodies, 2B Scientific, Upper Heyford, United Kingdom), LHCGR (LHR-N antibody; Eurogentec Ltd, Fawley, Southampton, United Kingdom), EGFR (sc-03; Santa Cruz Biotechnology, Santa Cruz, California), or CYP19A1 (PA1–21398; Thermo Scientific, Rockford, Illinois), incubating in the primary antibody for 90 minutes at room temperature (10); details of antibodies are provided in Supplemental Table 2. The blots were washed 3 times in Tris-buffered saline–Tween 20 and then were incubated for 60 minutes at room temperature in the appropriate secondary antibody (1:1000 in block solution IgG conjugated to horseradish peroxidase; Cell Signaling Technology, Danvers, Massachusetts). After a further 3 washes, protein reactivity was assessed using enhanced chemiluminescence (Luminata Forte Western HRP solution; Millipore Corporation, Billerica, Massachusetts). After imaging of each blot using the ChemiDoc XRS System (Bio-Rad), gels were stripped for 7 minutes in Restore Western Blot Stripping Buffer (Thermo Scientific) and then were reprobed for β-actin (1:1000, ab8226; Abcam, Cambridge, United Kingdom) to normalize protein loading. Images of at least 3 independent blots were analyzed for each protein using the ChemiDoc XRS System to measure peak density of each band, after adjustment for background (10).

Assessment of cell purity

Freshly isolated granulosa cells and PBMCs were examined for major histocompatability complex (MHC) II–positive cells using flow cytometry on 5 separate occasions. Initially, cells were incubated with 2 μM DRAQ5 (which binds DNA) for 30 minutes to gate for live DNA-containing cells. In a separate experiment, freshly isolated cells were incubated for 60 minutes at 4°C with mouse anti-ovine MHC II antibody (1:100 in fluorescence-activated cell sorting [FACS] buffer [0.2% BSA in PBS], bovine MHC II cross-reactive; AbD Serotec, Kidlington, United Kingdom) and then were washed 3 times in FACS buffer and incubated for 30 minutes at 4°C with goat anti-mouse Alexa 488 (1:500 in FACS buffer; Invitrogen) (10). Cells were washed 3 times in FACS buffer before analysis of 10,000 events using a FACSAria cell sorter (BD, San Jose, California). The cells were then washed twice with PBS, and mRNA was analyzed for *MHCII* and *CD45* expression by RT-PCR.

Statistics

Data are presented as arithmetic means and SEM. Statistical analyses were performed using SPSS version 16 (SPSS Inc Chicago, Illinois), and significance was ascribed when P values were $<.05$. ANOVA was used to examine normally distributed data, after square root or log transformation where indicated in *Results*, with the Dunnett pairwise multiple comparison t test used to compare treatments with control. Comparisons between treatment and control were made using the Mann-Whitney U test when data were not normally distributed, where indicated in *Results*. Because we were cognizant of between-animal biological variation for primary cell cultures, to specifically test whether siRNA targeting TLR2 or TLR4 or inhibitors of their downstream intracellular signaling pathways could reduce the IL-6 response to LPS or PAM, for each animal the concentrations of IL-6 were expressed as a percentage of the IL-6 accumulating in supernatants of cells treated with LPS or PAM, respectively.

Results

Granulosa cell cultures are free from immune cells and express TLR1–TLR10

There were no cells expressing MHC II in preparations of granulosa cells freshly isolated from dominant follicles, as determined by FACS analysis (0% positive cells vs 33% positive cells in blood-derived mononuclear cells) (Supplemental Figure 1A). Furthermore, the granulosa cells did not express mRNA for *CD45* or *MHCII*. However, as expected, the granulosa cells isolated from dominant follicles expressed *AMH, FSHR, LHCGR* and *CYP19A1* gene transcripts (Supplemental Figure 1B).

Freshly isolated granulosa cells expressed mRNA encoding all 10 TLRs examined by RT-PCR (Supplemental Figure 1C). The expression of mRNA encoding all 10 TLRs was still present in granulosa cells collected after 51 hours of culture in control medium (data not shown). Because bacterial infections perturb ovarian function (1–4), the present study focused on TLRs that recognize proto-

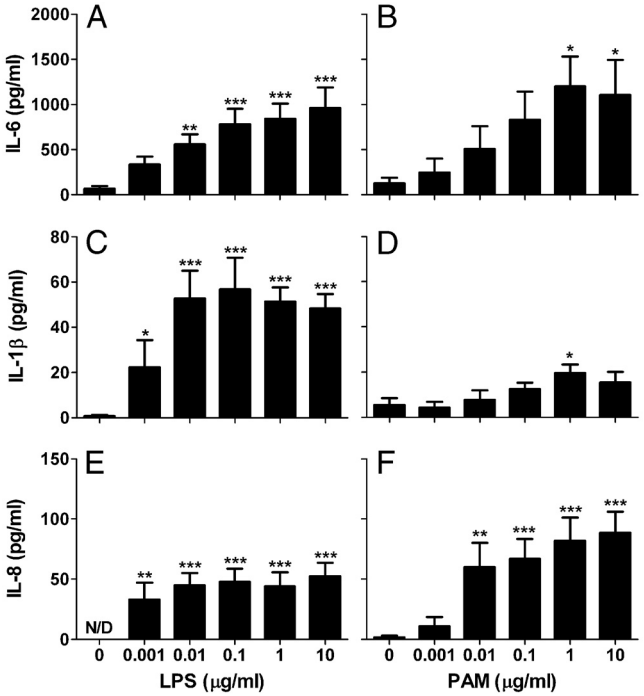

Figure 1. Granulosa cells responded to LPS or PAM. Granulosa cells isolated from dominant follicles were treated for 24 hours with control medium (0) or medium containing 0.001, 0.01, 0.1, 1, or 10 μg/mL LPS (A, C, and E) or PAM (B, D, and F). Supernatants were collected, and the accumulation of IL-6 (A and B), IL-1β (C and D), and IL-8 (E and F) was measured by ELISA. Data are presented as means + SEM from 3 independent experiments. Values differ from control by ANOVA and the Dunnett pairwise multiple comparison *t* test using $\log_{10}$-normalized data: *, $P < .05$, **, $P < .01$, ***, $P < .001$.

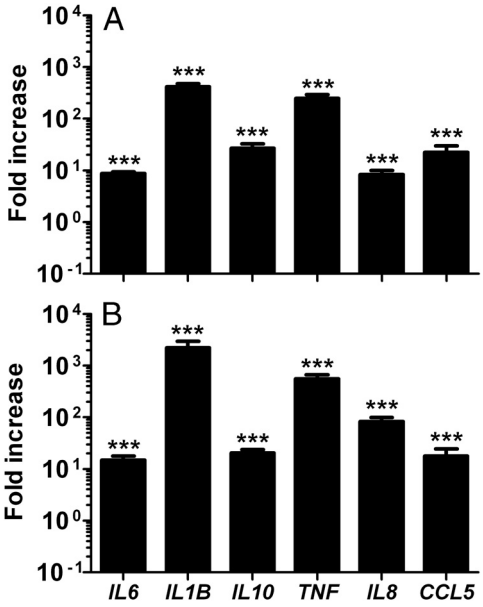

Figure 2. Granulosa cells increased expression of mRNA encoding inflammatory mediators in response to LPS or PAM. Granulosa cells isolated from dominant follicles were treated for 3 hours with control medium or medium containing 1 μg/mL LPS (A) or 1 μg/mL PAM (B), and the expression of genes encoding inflammatory mediators was examined by RT-PCR. Data are expressed relative to *ACTB* and are presented as mean fold change over control + SEM, from 4 independent experiments; values differ from the control by ANOVA and the Dunnett pairwise multiple comparison *t* test using $\log_{10}$-normalized data: ***, $P < .001$.

typical bacterial PAMPs: LPS bound by TLR4, bacterial lipoproteins via TLR2, and FLA bound by TLR5 (12, 13).

Granulosa cells respond to LPS and PAM

Granulosa cell supernatants accumulated IL-6, IL-1β, and IL-8 when treated for 24 hours with LPS (Figure 1, A, C, and E) or PAM (Figure 1, B, D, and F). However, even the highest concentration of 1 μg/mL FLA did not significantly increase accumulation of IL-6 above the control (88 ± 23 vs 31 ± 11 pg/mL), and the concentrations of IL-1β and IL-8 were below the limits of detection. None of the PAMPs stimulated the accumulation of TNFα (all samples below the limit of detection).

Granulosa cells increased expression of mRNA encoding the cytokines *IL1B, IL6, IL10*, and *TNF* and the chemokines *IL8* and *CCL5* after 3 hours of treatment with LPS (Figure 2A) or PAM (Figure 2B).

Granulosa cells respond to PAMPs via TLR pathways

To study the mechanisms underlying granulosa cell responses to PAMPs, subsequent experiments used the accumulation of IL-6 in response to 1 μg/mL LPS or PAM, because this provided a robust experimental paradigm and this LPS concentration reflects that previously reported in follicular fluid of cows with severe uterine disease (9). To examine the requirement for TLRs, granulosa cells were transfected with siRNA targeting *TLR4* or *TLR2*, which reduced the expression of *TLR4* and *TLR2* mRNA by 76% and 80%, respectively (Figure 3A). As expected, the supernatants of cells in vehicle accumulated IL-6 when treated with LPS (1580 ± 566 pg/mL [LPS] vs 266 ± 83 pg/mL [control medium]) or PAM (1814 ± 606 pg/mL [PAM] vs 232 ± 73 pg/mL [control medium]). However, cells transfected with siRNA targeting *TLR4* or *TLR2* had reduced accumulation of IL-6, expressed as a percentage of the IL-6 response within animals to LPS (Figure 3B) or PAM (Figure 3C), respectively, compared with granulosa cells treated with vehicle or scramble siRNA.

Intracellular signaling pathways associated with TLR activation were examined in granulosa cells by Western blotting for phosphorylated p38 and ERK protein (Figure 4, A and B). Granulosa cells showed little phosphorylation of p38 or ERK1/2 before exposure to PAMPs, but phosphorylated ERK1/2 was more abundant in cells 15 minutes after treatment with LPS (Figure 4C) and 10 minutes after treatment with PAM (Figure 4D). Similarly, phosphorylated p38 was more abundant in cells 15 minutes

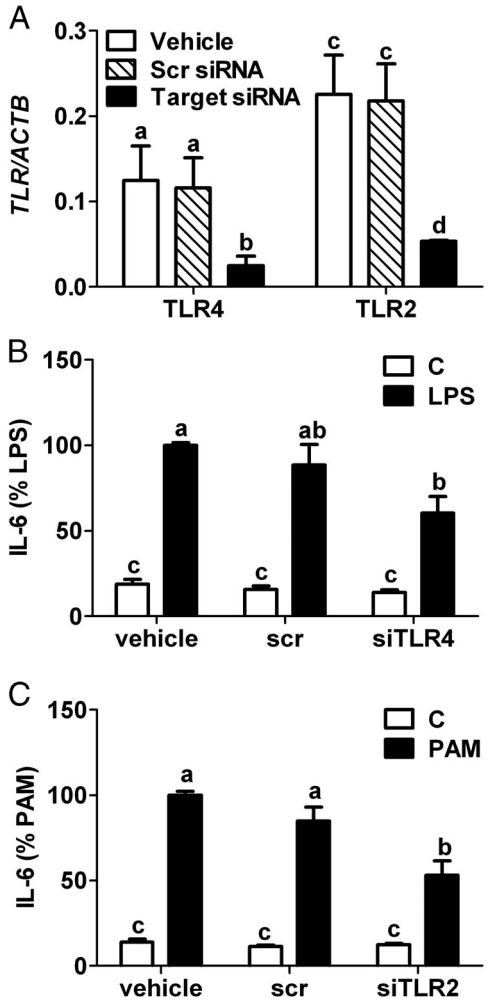

Figure 3. The IL-6 response of granulosa cells to LPS or PAM was attenuated using siRNA targeting *TLR4* or *TLR2,* respectively. A, Granulosa cells isolated from dominant follicles were treated for 24 hours with lipofectamine (open bars), nonspecific scramble siRNA (hatched bars), or specific siRNA (black bars) targeting *TLR4* or *TLR2*, and the expression of *TLR4* or *TLR2* was estimated by RT-PCR. Data are expressed relative to *ACTB* and are presented as means + SEM from 3 independent experiments. After 24 hours of treatment with lipofectamine (vehicle), scramble siRNA (scr), or siRNA targeting *TLR4* (siTLR4) or *TLR2* (siTLR2), granulosa cells were treated for a further 24 hours with control medium (open bars) or medium containing (solid bars) 1 μg/mL LPS (B) or 1 μg/mL PAM (C), and the accumulation of IL-6 was measured by ELISA and expressed as a percentage of the within-animal response to LPS or PAM, respectively. Data are presented as means + SEM, from at least 4 independent experiments for each siRNA. Values differing significantly by ANOVA ($\log_{10}$ normalized for RT-PCR data; square roots normalized for ELISA data) are denoted with different letters.

after treatment with LPS (Figure 4E) and 20 minutes after treatment with PAM (Figure 4F).

Activation of a specific TLR often modulates the expression of other TLRs (18). Treatment of granulosa cells for 3 hours with LPS significantly increased the expression of mRNA encoding *TLR2* and decreased the expression of *TLR4, TLR6, TLR7, TLR8*, and *TLR10* (Table 1). Similarly, treatment of granulosa cells for 3 hours with PAM also increased the expression of mRNA encoding *TLR2* and decreased the expression of *TLR6–TLR9* (Table 1). To test whether these changes in mRNA affected the production of proinflammatory cytokines, the granulosa cells were treated for a further 24 hours after the initial 3-hour pretreatment. Interestingly, pretreatment with LPS or PAM increased accumulation of IL-6 by granulosa cells subsequently treated with PAM for 24 hours (Supplemental Figure 2), mirroring the 3-fold increase in *TLR2* expression (Table 1).

Inhibiting NFκB, p38, or MEK limits IL-6 accumulation in response to PAMPs

To further explore which intracellular signaling pathways are mechanistically important for the innate immune response, granulosa cells were pretreated with inhibitors that target NFκB or specific MAPK before treatment with LPS or PAM. As expected, the supernatants of cells in vehicle accumulated IL-6 when treated with LPS (2031 $\pm$ 909 pg/mL [LPS] vs 360 $\pm$ 266 pg/mL [control medium]) or PAM (2158 $\pm$ 706 pg/mL [PAM] vs 290 $\pm$ 243 pg/mL [control medium]). There was evidence of reduced cell survival using the highest concentrations of inhibitors targeting NFκB, JNK, and p38 but not MEK (Figure 5). However, there was a reduction in the accumulation of IL-6 in culture supernatants in response to LPS, without a significant impact on cell survival at the concomitant concentration of inhibitors, when cells were pretreated with 0.04 to 4 μM NFκB inhibitor (Figure 5A), 5 μM JNK inhibitor (Figure 5B), 1 to 10 μM MEK inhibitor (Figure 5C), or 0.001 to 10 μM p38 inhibitor (Figure 5D). Similarly, there was a reduction in the accumulation of IL-6 in culture supernatants in response to PAM, without a significant impact on cell survival at the concomitant concentration of inhibitors, when cells were pretreated with 0.4 μM NFκB inhibitor (Figure 5E), 50 μM JNK inhibitor (Figure 5F), 1 to 10 μM MEK inhibitor (Figure 5G), or 1 to 10 μM p38 inhibitor (Figure 5H).

Exposure of granulosa cells to PAMPs affects endocrine function

To investigate the effect of PAMPs on endocrine function, granulosa cells were cultured in the presence of FSH and androstenedione, and the accumulation of estradiol and progesterone was measured in culture supernatants. Exposure to LPS or PAM reduced the accumulation of estradiol (Figure 6A) and progesterone (Figure 6B) in culture supernatants compared with that of cells in control medium, expressing the accumulation of each steroid as a percentage of control within animal. To explore which endocrine pathways may be important, granulosa cells were collected after 3 hours of treatment with LPS or PAM

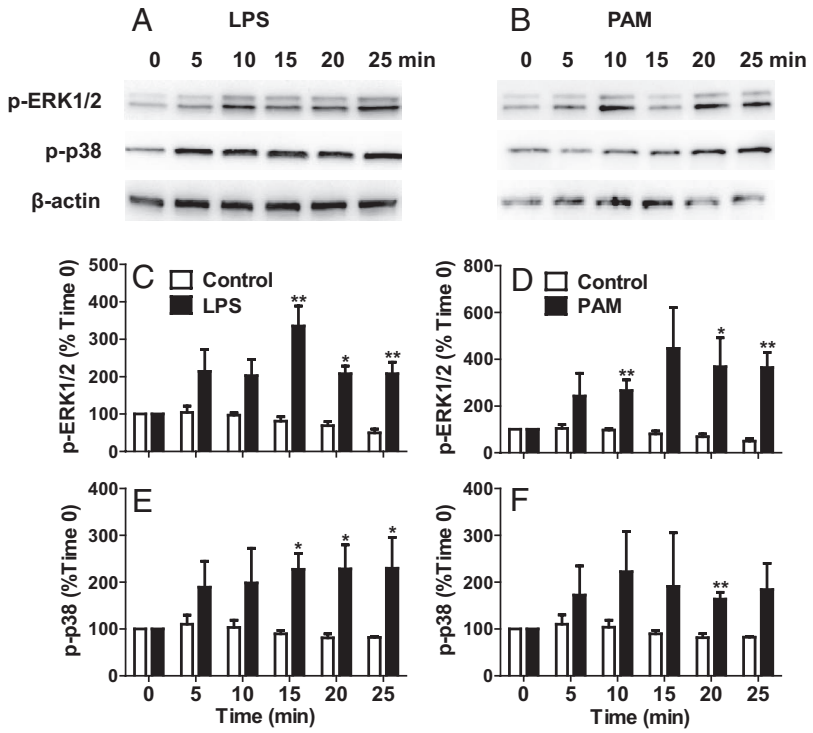

Figure 4. LPS or PAM induced phosphorylation of ERK1/2 and p38 in granulosa cells. Granulosa cells isolated from dominant follicles were treated for 0, 5, 10, 15, 20, or 25 minutes with control medium or medium containing 1 μg/mL LPS or PAM and phosphorylation of ERK1/2 and p38 was analyzed by Western blot. Representative images of Western blots after treatment with LPS (A) or PAM (B) are shown with bands corresponding to diphosphorylated ERK1/2, phosphorylated p38 (Thr180/182), and β-actin. Band densities were quantified relative to β-actin for phosphorylation of ERK1/2 (C and D) and p38 (E and F) and are expressed as a percentage of time 0 for cells in control medium (open bars) or treated (solid bars) with LPS (C and E) or PAM (D and F). Data are presented as means + SEM from 3 independent experiments. Values differ from control within time points by the Student *t* test ($\log_{10}$–normalized data): *, $P < .05$; **, $P < .01$.

to examine mRNA expression. Treatment of cells with LPS or PAM reduced the expression of mRNA encoding *CYP19A1* (Figure 6, C and D), although the effects on *LHCGR* and *EGFR* mRNA were less consistent and neither PAMP significantly changed *FSHR* mRNA expression. Although LHCGR and EGFR protein were not significantly affected, Western blotting confirmed that LPS and PAM markedly reduced the abundance of CYP19A1 (Figure 6, E and F).

Table 1. LPS or PAM Treatment Modulated the Expression of *TLR* Gene Transcripts

Gene	LPS	PAM
TLR1	0.90 ± 0.13	0.81 ± 0.08
TLR2	3.06 ± 0.79[a]	3.50 ± 1.06[a]
TLR3	2.01 ± 0.72	1.07 ± 0.17
TLR4	0.59 ± 0.05[b]	0.81 ± 0.15
TLR5	1.54 ± 0.29	0.70 ± 0.20
TLR6	0.65 ± 0.07[a]	0.64 ± 0.09[a]
TLR7	0.33 ± 0.03[b]	0.34 ± 0.06[b]
TLR8	0.24 ± 0.07[b]	0.30 ± 0.10[a]
TLR9	0.83 ± 0.09	0.56 ± 0.06[b]
TLR10	0.76 ± 0.01[b]	0.96 ± 0.24

Granulosa cells isolated from dominant follicles were treated for 3 hours with control medium or medium containing 1 μg/mL LPS or PAM. The expression of mRNAs encoding *TLR1–TLR10* was measured relative to *ACTB* by RT-PCR and is expressed as fold change over control. Data are presented as means ± SEM from 4 independent experiments.

Values differ from mRNA expression of cells in control medium by the Student *t* test using $\log_{10}$-normalized data: [a]$P < .05$; [b]$P < .01$.

Discussion

Bacterial infections of the uterus or mammary gland perturb ovarian dominant follicle growth and endocrine function (1, 3, 4). Although LPS has been found in follicular fluid from dominant follicles of animals with uterine disease (9), healthy ovarian follicles are devoid of immune cells (10, 11). The present study considered whether granulosa cells inside dominant follicles may have roles in innate immunity. Granulosa cells collected from dominant follicles expressed mRNA for all 10 TLRs and produced IL-1β, IL-6, and IL-8 in response to PAM and LPS via the TLR2 and TLR4 pathways, respectively. However, the cells did not accumulate cytokines in response to the TLR5 ligand FLA. Granulosa cells had rapid responses to PAM or LPS, with increased abundance of *IL6, IL1B, IL10, TNF, IL8*, and *CCL5* mRNA and increased phosphorylation of p38 and ERK1/2. Furthermore, treating granulosa cells with inhibitors targeting MAPK or NFκB reduced cellular responses to these PAMPs. Treatment with LPS or PAM also affected endocrine function by reducing the abundance of CYP19A1 and the accumulation of estradiol. Inflammatory responses to PAMPs via TLR2 and TLR4 pathways in granulosa cells provide a molecular explanation of how bacterial infections distant to the ovary may perturb ovarian dominant follicle function.

TLRs are conserved structures expressed in most animals, mainly by cells of the immune system such as macrophages and dendritic cells (12, 13, 19). However, a wider repertoire of cells have roles in innate immunity, including intestinal epithelial cells (20) and endometrium (21). Previous studies have identified expression of *TLR4* in bovine granulosa cells (9), *TLR4–TLR10* in a human granulosa cell line (22), and *TLR2* and *TLR4* in murine

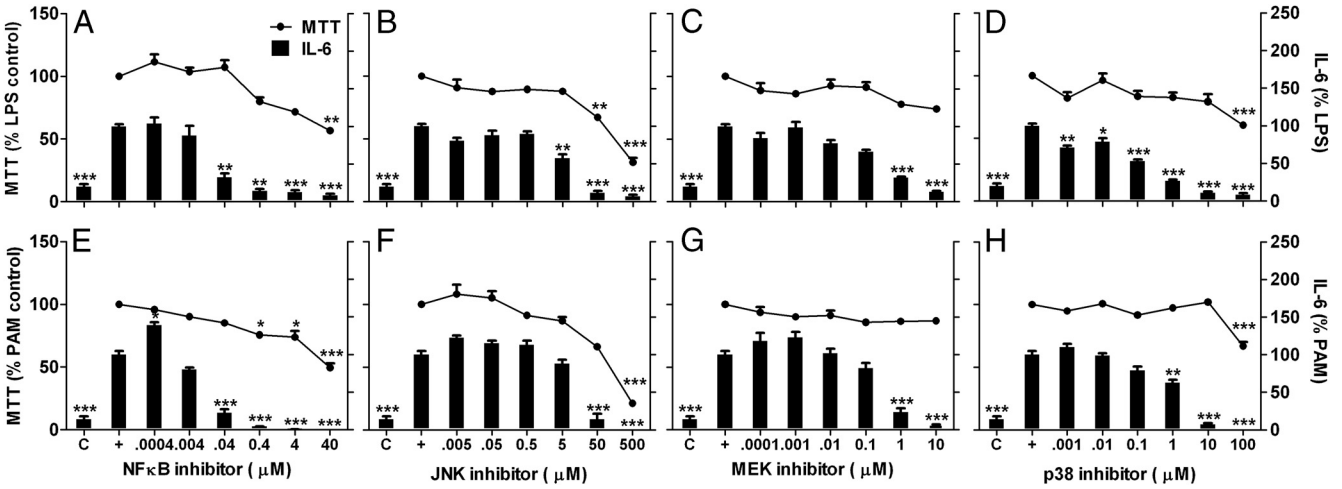

Figure 5. Inhibiting NFκB, p38, MEK, or JNK limited LPS- or PAM-induced IL-6 accumulation by granulosa cells. Granulosa cells isolated from dominant follicles were treated for 30 minutes with inhibitors targeting NFκB (A and E; 10-fold increasing concentrations from 0.0004 to 40 μM), JNK (B and F; 0.005–500 μM), MEK (C and G; 0.0001–10 μM), or p38 (D and H; 0.001–100 μM); followed by addition of control medium (C) or medium containing LPS (+; A–D) or PAM (+; E–H) to each well, making a final concentration of 1 μg/mL for 24 hours. Supernatants were collected, and the accumulation of IL-6 was measured by ELISA and is expressed as a percentage of the within-animal response to LPS or PAM, respectively (black bars). Cell viability was assessed by the MTT assay and is expressed as a percentage of the within-animal number of cells in the control medium (black circles). Data are presented as means + SEM, from at least 4 independent experiments for each inhibitor. Values differ from LPS or PAM treatment by ANOVA and the Dunnett pairwise multiple comparison *t* test (E–H; $\log_{10}$–normalized data for MTT and square root–normalized data for IL-6): *, $P < .05$; **, $P < .01$; ***, $P < .001$.

granulosa cells (23). In the present study, FACS and PCR analysis were used to confirm that the preparations of granulosa cells isolated from dominant follicles were free of immune cell contamination, as determined by lack of *CD45* and *MHCII* expression, because such contaminations might have confounded the investigations. The bovine granulosa cells from dominant follicles expressed mRNA encoding all 10 TLRs, and expression was maintained during cell culture. The granulosa cell expression of *TLR2*, *TLR4*, and *TLR5* mRNA was of immediate relevance because these receptors are required to detect bacterial PAMPs used in the present study. Pretreatment of granulosa cells with LPS or PAM increased both expression of *TLR2* and cellular responses to further PAM treatment, suggesting that PAMPs have the ability to prime granulosa cells to respond to other TLR ligands. Supporting this hypothesis that LPS primes cells to respond to TLR2 ligands, LPS pretreatment also increases IL-6 accumulation by murine bone marrow–derived macrophages subsequently treated with the TLR2/TLR6 agonist FSL-1 (24), and pretreatment of dendritic cells with LPS increases IL-6 production by cells subsequently challenged with PAM (25).

The supernatants of granulosa cells from dominant follicles treated with LPS or PAM for 24 hours accumulated the cytokines IL-1β and IL-6 and the chemokine IL-8. In addition to protein accumulation, the granulosa cells mounted acute responses after 3 hours of treatment with LPS and PAM, characterized by increased expression of gene transcripts for the cytokines *IL6, IL1B, IL10*, and *TNF* and the chemokines *IL8* and *CCL5*. The changes in inflammatory mediators found in the present study are typical of responses to LPS or PAM by macrophages and dendritic cells (12, 13, 19). Interestingly, many inflammatory mediators associated with innate immunity also have physiological functions in the ovary. For example, IL-6 induces murine cumulus-oocyte complex expansion (26) and IL-1β decreases progesterone production by FSH-treated murine granulosa cells (27). Chemokines also play a role, with IL-8 involved in follicle development, ovulation, and corpus luteum formation (28). The absence of TNFα protein in the present study might be considered surprising for some tissues and immune cells; however, changes in protein are less often detected than changes in *TNF* gene expression in cells of the bovine reproductive tract (29). The importance of TLR2 and TLR4 in bovine granulosa cells was confirmed using siRNA, which reduced *TLR2* mRNA expression by 76% and *TLR4* mRNA expression by 80%, leading to accumulation of less IL-6 in the supernatants of granulosa cells treated with PAM or LPS. However, IL-6 accumulation was not abrogated by siRNA, which probably reflects the relatively few receptors required to trigger innate immunity; occupation of 50 to 100 TLR4 is sufficient to trigger measurable TLR4-dependent cell activation and <20% receptor occupancy gave maximal activation (30). Although TLR2 has not been investigated previously, bovine granulosa cells collected from small emerged follicles before domi-

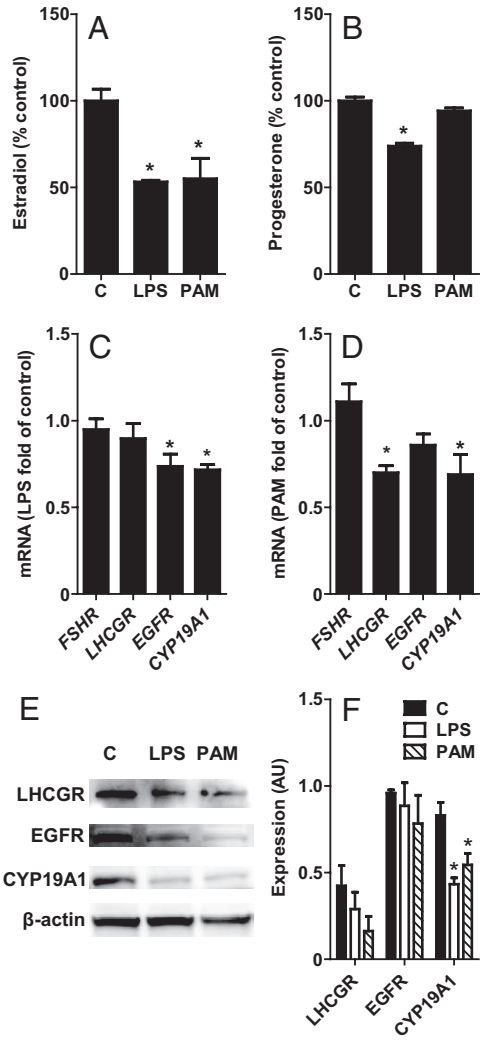

Figure 6. Granulosa cell endocrine function is affected by LPS or PAM. Granulosa cells were cultured for 24 hours with control medium containing 1 ng/mL FSH and 10^{-7} M androstenedione (C) or medium containing 1 μg/mL LPS or 1 μg/mL PAM in addition to 1 ng/mL FSH and 10^{-7} M androstenedione. Supernatants were collected, and the accumulation of estradiol (A) and progesterone (B) was measured by ELISA and expressed as a percentage of control within animal. Data are presented as means + SEM from 3 independent experiments; values differ from control by Mann-Whitney *U* test: *, $P < .05$. Granulosa cells isolated from dominant follicles were treated for 3 hours with control medium or medium containing 1 μg/mL LPS (C) or 1 μg/mL PAM (D), and the expression of genes encoding endocrine receptors was examined by RT-PCR. Data are expressed relative to *ACTB* and are presented as mean + SEM fold change over control from 4 independent experiments. Values differ from control by ANOVA and the Dunnett pairwise multiple comparison *t* test using log-normalized data: *, $P < .05$. E, Granulosa cells isolated from dominant follicles were treated for 24 hours with control medium or medium containing 1 μg/mL LPS or PAM and analyzed by Western blot. Images of Western blots for one experiment, representative of 4 independent experiments are shown with bands corresponding to CYP19A1, EGFR, LHCGR, and β-actin. F, Band densities were quantified relative to β-actin and are expressed as arbitrary units for cells in control medium (solid bars), LPS (open bars), and PAM (hatched bars). Data are presented as means + SEM from 4 independent experiments. Values differ from control by the Mann-Whitney *U* test: *, $P < .05$.

nance also required TLR4 to respond to LPS (10). A remaining question is how PAMPs from bacteria in the uterus, mammary gland, or other tissues may reach the ovarian follicular fluid and granulosa cells. Although follicular fluid collected in vivo from normal postpartum animals has no detectable LPS, between 4.3 and 875.2 ng/mL LPS has been found in the follicular fluid of cows with uterine disease (9). However, it remains unclear which local, lymphatic, or vascular routes are important for PAMPs to reach the dominant follicle.

Unfortunately, there is little consistent evidence that a reduction in systemic inflammation by clinical use of nonsteroidal anti-inflammatory agents improves ovarian function or fertility in postpartum cattle, with or without uterine disease (31). In immune cells, binding of bacterial PAMPs to TLRs activates NFκB and MAPK pathways, leading to production of inflammatory mediators (19). In the present study, treatment of granulosa cells from dominant follicles with LPS or PAM also increased ERK1/2 and p38 phosphorylation within 20 minutes; similar to bovine granulosa cells collected from follicles before dominance and treated with LPS (10). To extend these findings, in the present study, inhibitors targeting NFκB, JNK, MEK, or p38 that reduced the TLR4 or TLR2-mediated inflammatory response by granulosa cells were identified.

Cows with uterine disease or mastitis have lower peripheral plasma estradiol concentrations and delayed ovulation in vivo (2, 4). The accumulation of estradiol and progesterone is decreased when granulosa cells from emerged or dominant follicles are treated with 1 μg/mL LPS (9). Similarly, in the present study, there was an $\sim$50% decrease in estradiol accumulation in granulosa cells from dominant follicles after treatment with LPS or PAM. The reduced secretion of estradiol is probably explained in the present study by the decreased *CYP19A1* mRNA expression and lower CYP19A1 protein abundance in granulosa cells treated with LPS or PAM. It is likely that these changes in CYP19A1 would have a detrimental impact on follicle growth and the likelihood of ovulation because aromatization of androstenedione to estradiol is central to ovarian follicle function (6).

In conclusion, bovine granulosa cells from dominant follicles expressed functional TLR4 and TLR2 but not TLR5. Granulosa cells produced cellular responses to LPS and PAM, with increased expression and accumulation of inflammatory cytokines and chemokines, activation of MAPK pathways, and perturbation of steroidogenesis. The present study supports the hypothesis that granulosa cells from dominant follicles play a role in innate immunity in the ovary.

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Address all correspondence and requests for reprints to: Professor Iain Martin Sheldon, Institute of Life Science, School of Medicine, Swansea University, Singleton Park, Swansea, SA2 8PP, United Kingdom. E-mail: i.m.sheldon@swansea.ac.uk.

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