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

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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-κB 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)
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) (5). 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 (DPBS) (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)
hemin, 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 × 10^5 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 × 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 again, resuspended at 1 × 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; 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 × 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 PAMs
To evaluate granulosa cell responses to PAMs, 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 typhimu-rium (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, GGA-CAGAAUAGACACCAGUAU; antisense, UAGGUGUCUAU-UCUGUCCUU) or TLR4 (sense, GAGGUAUAUCUUAG- GAAGUUU; antisense, ACUCUCCUAAGAUCCUUCUU), 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 dis- solved in DMSO (maximal concentration of 1:500), and treatments spanned 10-fold increasing concentrations of inhibitors targeting NFKB (NFKB activation inhibitor InSolution, final concentra- tion ranging from 0.0004 to 40 μM; Calbiochem, San Diego, California), JNK (JNK inhibitor II, 0.005–500 μM; Cal- biochem), p38 (SB230580, 0.001–100 μM; Calbiochem), or mito- togen-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 pro- gesterone, 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, superna- tants 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 spectropho-tometer (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, inflammmatory 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), gels were stripped for 7 minutes in Restore Western Blot Stripping Buffer (Thermo Scientific) and then were reprobed (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 histocompatibility 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 fluorescein-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-
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...
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 or PAM (2158 ± 706 pg/mL [PAM] vs 290 ± 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.
endometrium (9), including intestinal epithelial cells (20) and endometrium 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 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).

**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

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**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 D) 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 (log10-normalized data): *, P < .05; **, P < .01.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>LPS</th>
<th>PAM</th>
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<tbody>
<tr>
<td>TLR1</td>
<td>0.90 ± 0.13</td>
<td>0.81 ± 0.08</td>
</tr>
<tr>
<td>TLR2</td>
<td>3.06 ± 0.79a</td>
<td>3.50 ± 1.06a</td>
</tr>
<tr>
<td>TLR3</td>
<td>2.01 ± 0.72</td>
<td>1.07 ± 0.17</td>
</tr>
<tr>
<td>TLR4</td>
<td>0.59 ± 0.05b</td>
<td>0.81 ± 0.15</td>
</tr>
<tr>
<td>TLR5</td>
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<td>0.70 ± 0.20</td>
</tr>
<tr>
<td>TLR6</td>
<td>0.65 ± 0.07a</td>
<td>0.64 ± 0.09a</td>
</tr>
<tr>
<td>TLR7</td>
<td>0.33 ± 0.03b</td>
<td>0.34 ± 0.06b</td>
</tr>
<tr>
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<td>0.30 ± 0.10a</td>
</tr>
<tr>
<td>TLR9</td>
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</tr>
<tr>
<td>TLR10</td>
<td>0.76 ± 0.01b</td>
<td>0.96 ± 0.24</td>
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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 log10-normalized data: *P < .05; **P < .01.
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 and cellular responses to further PAM treatment (24), and pretreatment of dendritic cells with LPS increased IL-6 accumulation by murine bone marrow–derived macrophages (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 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 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; log10–normalized data for MTT and square root–normalized data for IL-6): *, P < .05; **, P < .01; ***, P < .001.

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).
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 ANOVA and the Dunnett pairwise multiple comparison test using log-normalized data: *, P < .05. Estradiol (% control) and progesterone (% control) are presented as means ± SEM from 3 independent experiments. Values differ from control by ANOVA and the Dunnett pairwise multiple comparison test: *, P < .05. 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.

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 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.

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Acknowledgments

We thank Dr. Cathy Thornton for assistance with FACS, Dr. James Cronin for assistance with Western blotting, and Prof. Venkateswarlu Kanamalapudi for the LHCGR antibody.

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This work was supported by the Biotechnology and Biological Sciences Research Council (Grant BB/G017131/1).

Disclosure Summary: The authors have nothing to disclose.

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