Lipopolysaccharide Initiates Inflammation in Bovine Granulosa Cells via the TLR4 Pathway and Perturbs Oocyte Meiotic Progression *in Vitro*

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Infections of the reproductive tract or mammary gland with Gram-negative bacteria perturb ovarian function, follicular growth, and fecundity in cattle. We hypothesized that lipopolysaccharide (LPS) from Gram-negative bacteria stimulates an inflammatory response by ovarian granulosa cells that is mediated by Toll-like receptor (TLR) 4. The present study tested the capacity of bovine ovarian granulosa cells to initiate an inflammatory response to pathogen-associated molecular patterns and determined subsequent effects on the *in vitro* maturation of oocytes. Granulosa cells elicited an inflammatory response to pathogen-associated molecular patterns (LPS, lipoteichoic acid, peptidoglycan, or Pam3CSK4) with accumulation of the cytokine IL-6, and the chemokine IL-8, in a time- and dose-dependent manner. Granulosa cells responded acutely to LPS with rapid phosphorylation of TLR signaling components, p38 and ERK, and increased expression of *IL6* and *IL8* mRNA, although nuclear translocation of p65 was not evident. Targeting *TLR4* with small interfering RNA attenuated granulosa cell accumulation of IL-6 in response to LPS. Endocrine function of granulosa cells is regulated by FSH, but here, FSH also enhanced responsiveness to LPS, increasing IL-6 and IL-8 accumulation. Furthermore, LPS stimulated IL-6 secretion and expansion by cumulus-oocyte complexes and increased rates of meiotic arrest and germinal vesicle breakdown failure. In conclusion, bovine granulosa cells initiate an innate immune response to LPS via the TLR4 pathway, leading to inflammation and to perturbation of meiotic competence. (**Endocrinology** 152: 5029–5040, 2011)

Bacterial infections of the uterus or mammary gland commonly cause disease in dairy cattle, and these infections are associated with infertility (1–4). Uterine infection after parturition causes metritis in 40% of animals, and the resulting decreased milk yields, delayed ovulation, reduced fecundity, and animal culling for failure to conceive cost the European Union dairy industry €1.4 billion/yr. Infection of the mammary gland causes mastitis in a comparable proportion of animals, and these infections reduce conception rates. Metritis or mastitis also retard follicular growth, reduce circulating and intrafollicular estradiol concentrations, extend luteal phases, and disrupt ovarian cyclic activity (5–7). The Gram-negative bacterium *Escherichia coli* is a main pathogen causing metritis and mastitis, and these infected animals have reduced fecundity even after resolution of clinical disease (8, 9). Accumulation of lipopolysaccharide (LPS) from Gram-negative bacteria in follicular fluid of animals with metritis may link infection and ovarian dysfunction (2). Estradiol is reduced in granulosa cells cultured with LPS (3), whereas animals with mastitis have altered granulosa cell gene expression and lower follicular estradiol (4). Bacterial infections of the uterus in women also cause infertility (6, 10). Recently, microbial colonization and altered cytokine profiles were reported in follicular fluid from *in vitro* fertilization patients with low conception rates (11).

Abbreviations: CD, Cluster of differentiation; COC, Cumulus-oocyte complex; FCS, fetal calf serum; IκBα-EGFP, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α-enhanced green fluorescent protein; IVM, *in vitro* maturation; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MHCII, major histocompatibility complex class II; NFκB, nuclear factor κB; Pam3CSK4, N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine; PAMP, pathogen-associated molecular pattern; PGN, peptidoglycan; siRNA, small interfering RNA; TLR, Toll-like receptor.

However, mechanisms linking bacterial infection and perturbation of ovarian function or oocyte quality remain to be determined.

The Toll-like receptors (TLR) are a family of 10 cellular receptors responsible for detecting and initiating the innate immune defense against bacterial, viral, and fungal pathogens (12, 13). These receptors are primarily found on immune cells, such as macrophages, and generate the initial inflammatory response to a pathogen by binding pathogen-associated molecular patterns (PAMPs). LPS derived from *E. coli* is a prototypical PAMP binding TLR4 in complex with coreceptors cluster of differentiation (CD) 14 and myeloid differentiation factor-2, resulting in phosphorylation of ERK1/2 and p38, and nuclear translocation of nuclear factor κB (NFκB) components, which leads to production of proinflammatory cytokines, such as IL-1β, IL-6, TNFα, and chemokines, such as IL-8 (12, 13). Bovine and murine granulosa cells also express mRNA for the TLR4 receptor complex (2, 14). It remains unclear whether granulosa cells respond to LPS via TLR4 to generate an inflammatory response akin to cells of the immune system. This is important, because although ovarian stroma contains immune cells for tissue remodeling, healthy follicles are devoid of immune cells (15).

Mammalian oocyte growth and maturation from the primordial follicle until ovulation is dictated by a highly ordered cascade of hormones, growth factors, nutrients, and signaling molecules from the surrounding environment (16, 17). Oocytes must undergo nuclear and cytoplasmic maturation for successful fertilization and embryonic development, progressing from the germinal vesicle stage until pausing at the M-phase of meiosis II (18). Oocytes depend on their surrounding granulosa cells for nutrition, and there is bidirectional communication between oocyte and granulosa cells. However, these intimate interactions expose mammalian oocytes to more exogenous factors than invertebrate eggs enclosed in an impermeable shell. Thus, in the absence of immune cells in the ovarian follicle, perhaps granulosa cells play an active role in protecting mammalian oocytes against PAMPs. Although mice with defective TLR4 signaling have normal fertility (19, 20), TLR2 and TLR4 complexes binding endogenous ligands, such as hyaluronic acid in ovulated cumulus-oocyte complexes (COCs), play a role in sperm capacitation and oocyte fertilization (21). Ovulation itself is regarded as sterile inflammation involving the innate immune system (22, 23). However, it is not clear whether during disease the activation of TLR4 by LPS could impact oocyte competence during follicle development.

Here, we explore the mechanism of ovarian perturbation associated with PAMPs and investigate the possibility that granulosa cells act as immune sensors within the ovarian follicle. We tested the capacity of bovine ovarian granulosa cells to initiate an inflammatory response to PAMPs and determined subsequent effects on the *in vitro* maturation (IVM) of oocytes. Here, we show that *in vitro* exposure of granulosa cells and oocytes to LPS generates a TLR4-dependent inflammatory response and ultimately perturbations in oocyte meiotic competence.

Materials and Methods

Tissue collection and cell isolation

Ovaries were collected from cows within 15 min of killing and transported to the laboratory on ice in PBS containing 1% penicillin/streptomycin (Sigma-Aldrich, Poole, UK). Ovaries from between 10 and 20 cows were pooled for each experiment. Within 90 min of excision, ovaries were processed for collection of mural granulosa cells and COCs. Ovaries were rinsed in 70% ethanol followed by a brief rinse in sterile PBS. An endotoxin-free 2-ml syringe and 20-gauge needle was employed to aspirate 4- to 8-mm follicles into collection medium [Medium 199 (Invitrogen, Paisley, UK), 0.5% BSA, 25 mM HEPES, 50 μg/ml heparin, 5 U penicillin, and 50 μg/ml streptomycin; Sigma-Aldrich]. The 4- to 8-mm follicles were chosen because they are a representative homogenous pool of follicles with granulosa cells that are FSH responsive and LH unresponsive (24). The COCs were collected and pooled for two 5-min washes in fresh collection medium without heparin and then placed into defined maturation culture medium (see below). Granulosa cells were pooled and washed twice in either serum-free medium as previously defined (25) or granulosa cell culture medium containing 10% fetal calf serum (FCS). Granulosa cells were then resuspended and plated at 1.5×10^6 cells/ml in a final volume of 500 μl in 24-well plates (TPP, Trasadingen, Switzerland). Each experiment was carried out at least four times using tissue collected on different days. Treatments were performed in single wells with negative and positive controls.

Assessment of immune cells

Independent granulosa and blood cell cultures on three separate occasions were subjected to mRNA extraction for RT-PCR or fixation for immunocytochemistry (see below). Freshly isolated granulosa cells were also subjected to flow cytometry analysis by incubating with mouse antiovine major histocompatibility complex class II (MHCII) antibody (1:100, bovine MHCII cross-reactive; AbD Serotec, Kidlington, UK). Cells were incubated for 1 h on ice in PBS and 0.2% BSA (Sigma-Aldrich), washed three times in fresh PBS and 0.2% BSA, incubated for 30 min with goat antimouse Alexa Fluor 488 (1:500; Invitrogen), washed in PBS and 0.2% BSA three times, and 10000 events analyzed using FACSAria (BD Biosciences, San Jose, CA). The proportion of MHCII positive cells was determined by plotting against the unstained population.

Granulosa cell PAMP challenge

After an initial establishment period of 48 h, cultured granulosa cell supernatants were replaced with fresh medium containing the following PAMPs: ultrapure LPS (ligand for TLR4),

lipoteichoic acid (LTA) (ligand for TLR2), peptidoglycan (PGN) (ligand for TLR2), or Pam3CSK4 (N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine) (synthetic ligand for TLR1 and TLR2) at 10-fold increasing doses between 100 pg/ml and 10 μg/ml (all InvivoGen, San Diego, CA). Cell-free supernatants were collected 24 or 48 h after treatment and stored at -20 C for analysis by ELISA. Each experiment was repeated on four to six separate occasions.

To determine time-dependent activation of granulosa cells in response to LPS, cells were treated with 1 μg/ml LPS for 0, 30, 60, 90, 180 min. At the end of each experiment, the cells were collected and total mRNA or protein was isolated (see below). Each experiment was repeated on four separate occasions.

Oocyte IVM

COCs were pooled and randomly assigned to undergo IVM in different conditions for 24 h. Meiotic evaluation was performed on three independent occasions, IL-6 accumulation on four occasions, and COC expansion on eight occasions. Maturation medium (Medium 199, 0.25 mM pyruvate, 10% FCS, 1 μg/ml estradiol, 1% insulin/transferrin/sodium selenite solution, 5 U penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine) was supplemented with gonadotropins using 2.5 μg/ml FSH and/or 10 μg/ml LH (both A. F. Parlow, National Hormone and Peptide Program, Torrance, CA) (26, 27) and LPS at 1 or 10 μg/ml. The COCs were cultured in 1 ml of defined medium in round bottomed organ culture dishes in groups of 10–15 per experiment. After IVM, COC expansion was recorded, cell-free supernatants collected, and COCs partially denuded in 10 IU hyaluronidases (Irvine Scientific, Wicklow, Ireland) ready for meiotic evaluation by confocal microscopy.

Tissue fixation, immunofluorescence, and confocal microscopy

Cultured granulosa cells or COCs were fixed in 2% paraformaldehyde for 10 min at 37 C followed by microtubule stabilizing buffer (100 mM PIPES, 5 mM MgCl$_2$, 2.5 mM EGTA, 2% formaldehyde, 0.1% Triton X-100, 1 mM taxol, and 10 U/ml aprotinin; Sigma-Aldrich) for 45 min at 37 C and stored in wash solution, as described previously (28, 29). Samples were incubated overnight in the presence of an antibody cocktail of mouse anti-α-tubulin and mouse anti-β-tubulin to assess meiotic progression (1:100; Sigma-Aldrich) or ovine anti-MHCII to evaluate the presence of immune cells (1:50; AbD Serotec) at 4 C. Samples were washed three times in wash solution for a total of 30 min, and antibody detection was performed using goat anti-mouse-Alexa Fluor 488 secondary antibody (1:800; Invitrogen), in combination with Phalloidin-Alexa Fluor 555 (1:100; Invitrogen) for the detection of F-actin, and 1 μg/ml Hoechst 33342 (Invitrogen) at 37 C with gentle agitation. Samples were washed three times in wash solution for a total of 30 min before mounting in 50% glycerol/PBS using wax cushions to avoid compression of samples (28, 29). Ovary or spleen were fixed in 4% paraformaldehyde overnight, wax embedded, and subjected to immunohistochemistry to identify the presence of immune cells by MHCII immunoreactivity.

Samples were analyzed on a Zeiss LSM 710 confocal microscope using a $\times$40 Plan-Apochromat objective (na = 1.3), KrArg (405,488 nm), and HeNe (543 nm) lasers to collect three channel z-stacks through the entire spindle of each oocyte using Zen software (Zeiss, Jena, Germany). Oocytes were categorized by evaluating chromatin condensation, cortical actin arrangement, spindle bipolarity, and presence of a polar body; only oocytes with a polar body and bipolar spindle with condensed chromatids were considered as normal MII oocytes (29). Oocytes were evaluated by an experienced observer without reference to treatment group.

RNA isolation and real-time RT-PCR

Total RNA was isolated from cell samples after two washes in PBS. Samples were resuspended in RLT buffer (QIAGEN, Crawley, UK) 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 the NanoDrop spectrophotometer, and 2 μg of mRNA were subjected to RT using the QuantiTect Reverse Transcription kit (QIAGEN) according to the manufacturer's instructions. Primers were designed using the NCBI database and initial specificity verified by BLAST to ensure no cross-reactivity with other loci (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Real-time PCR was performed in 25-μl reactions containing 1 μM each forward and reverse primer (Sigma Genosys). An iQ5 light cycler (Bio-Rad, Hemel Hempstead, UK) was employed to perform quantitative PCR. The starting quantity of mRNA from each sample was determined using standard curves generated from reference RNA with Quantifast SYBR green (QIAGEN), and expression levels of genes of interest were then normalized to the reference gene *ACTB* after verification of stable expression (30). To examine cell purity, PCR products for *MHCII* and *AMH* were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized under UV illumination.

Protein isolation and Western blotting

Total protein was isolated from cells after two washes in PBS. A total of 10 μg of protein was electrophoresed on a 10% SDS-PAGE. Protein was transferred onto polyvinylidene fluoride membranes and blocked overnight in 5% BSA in Tris-buffered saline with Tween 20 at 4 C. Protein blots were probed for diphosphorylated-ERK1/2 (M8159; Sigma-Aldrich) and phosphorylated-p38 (Thr180/Tyr182; Acris Antibodies, Herford, Germany). Membranes were incubated for 2 h at room temperature with the appropriate primary antibody diluted 1:1000 in block solution. After three 15-min washes in Tris-buffered saline with Tween 20, membranes were incubated with an appropriate secondary antibody conjugated to horseradish peroxidise diluted 1:1000 in block solution for 2 h (Cell Signaling, Danvers, MA). After three washes, protein reactivity was visualized using enhanced chemiluminescence (Western C; Bio-Rad). Protein loading was normalized to tubulin immunoreactivity (Invitrogen) performed on the same blot.

Small interfering RNA (siRNA)

Inhibition of TLR4 mRNA translation was performed using siRNA targeted to *TLR4* mRNA (Fisher Scientific, Loughborough, UK) (Supplemental Table 1). Cells were incubated with Lipofectamine-RNAiMAX (Invitrogen) and 10 pmol TLR4-

siRNA or scramble-siRNA (Fisher Scientific) in the absence of antibiotics for a period of 24 h. After washing in PBS, cells were challenged with 1 μg/ml LPS for an additional 24 h. Cell-free supernatant and total mRNA were collected for analysis. Experiments were performed on four separate occasions.

Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α-enhanced green fluorescent protein (IκBα-EGFP) and p65-dsRed cell transfection for time-lapse confocal microscopy

The transcription factor p65 remains inactive when sequestered by IκBα in the cytoplasm, but upon IκBα ubiquitination and degradation, p65 is free to translocate to the nucleus (31, 32). To determine IκBα degradation and p65 translocation, granulosa cells were cultured on four chamber glass bottom dishes at 10^4 cells/ml for 24 h under normal culture conditions and then transfected with Lipofectamine 2000 (Invitrogen) and a plasmid containing IκBα-EGFP and a p65-dsRed plasmid, both under control of the hCMV-IE promoter (gift from Dr. Violaine Sée, University of Liverpool, Liverpool, UK) (31, 32). Transfection of plasmids was initially performed in complete granulosa cell medium in the absence of antibiotics. After 6 h of culture, cells were washed in PBS and cultured for a further 18 h in serum-free granulosa cell medium to facilitate p65 localization to the cytoplasm before imaging. All microscopy was carried out in complete granulosa cell culture medium containing serum, and cells were placed in control medium or media containing 1 μg/ml LPS, or 20 ng/ml TNFα as a positive control. Cells were visualized using multiplexed, time-lapse confocal microscopy with an automated stage in a live cell chamber (Zeiss 710 LSM) maintained at 37 C with 5% CO_2 in air allowing visualization of control and treated cells contemporaneously. Experiments were carried out on three separate occasions with a minimum of five cells visualized per treatment for each experiment.

Enzyme-linked immunosorbent assay

IL-6 and IL-8 were measured in cell-free supernatants using commercially available ELISA [Fisher Scientific and R&D Systems (Abingdon, UK), respectively] according to the manufacturer's instructions. Although the IL-6 ELISA is bovine specific, the IL-8 ELISA is cross-reactive for bovine IL-8 (33).

Statistics

SPSS version 13.0 was used for statistical analysis. Data are presented as the mean + SEM. Real-time RT-PCR and gonadotropin experiments were analyzed using a nonparametric Mann-Whitney U test. The PAMP challenge experiments were analyzed using ANOVA followed by Dunnett's pair-wise *post hoc* test. IL-6 data for COCs were first log transformed to normalize data distribution before ANOVA followed by Dunnett's pair-wise *post hoc* test. Meiotic failure of oocytes was compared using the χ^2 test. $P < 0.05$ was assumed to be statistically significant.

Results

Granulosa cells are free of immune cells

Contamination of cultured granulosa cells with immune cells was assessed to ensure that inflammatory re-

sponses were due to nonimmune cells. Previous work showed that granulosa cell cultures do not express the CD 45 immune cell marker (2). In the present study, the MHCII marker of antigen presenting cells was not expressed by granulosa cell cultures (Fig. 1A). Also, cultured granulosa and blood cells had 0 and 71.4% MHCII positive cells, respectively (Fig. 1B). Flow cytometry analysis of freshly isolated granulosa and blood cells had 0 and 44% MHCII positive cells, respectively (Fig. 1C). Furthermore, MHCII immunoreactive cells were absent within the basement membrane of intact 4- to 8-mm follicles (Fig. 1D).
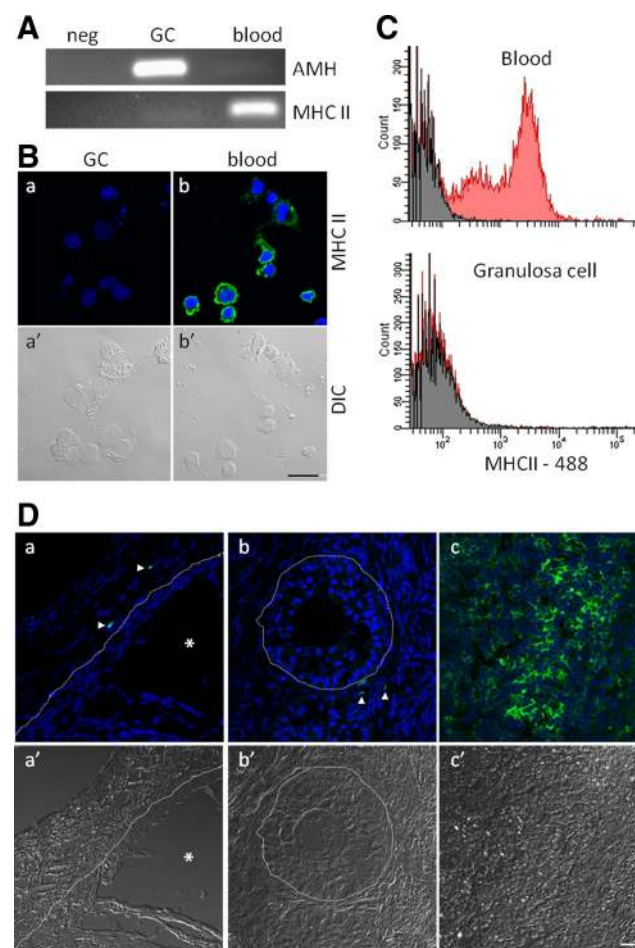

FIG. 1. Granulosa cell cultures are free of immune cells. Cultured granulosa cells were assessed for contamination with immune cells. A, PCR amplification of MHCII and anti-Müllerian hormone (AMH) in cultured granulosa and blood cells. B, Confocal micrographs of cultured granulosa (a) and blood (b) cells showing MHCII immunoreactivity (*green*), DNA (*blue*), and DIC (a' and b'). C, Flow cytometry histograms of freshly isolated blood and granulosa cells showing MHC reactivity in *red* and unstained cells in *gray*. D, Confocal micrographs representing a cross section of a 4- to 8-mm follicle (a), a less than 4-mm follicle (b) and spleen (c) showing MHCII immunoreactivity (*green*), DNA (*blue*), and DIC (a'–c'). *Arrowheads* indicate MHCII positive cells, the *asterisk* represents the antral cavity, and the *dashed line* represents the basement membrane extrapolated from the DIC image. *Scale bar*, 20 μm. DIC, Differential interference contrast: GC, granulosa cell; neg, negative.
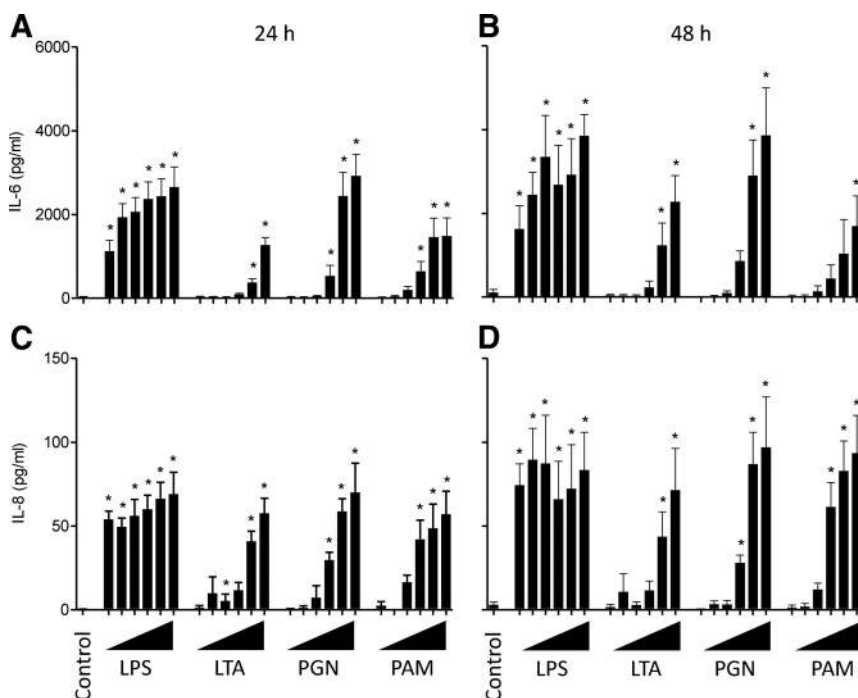
FIG. 2. Granulosa cells produced IL-6 and IL-8 in response to LPS, LTA, PGN, and Pam3CSK4 (PAM) in a dose-dependent manner. Accumulation of IL-6 (A and B) and IL-8 (C and D) in the supernatants of granulosa cells was measured by ELISA after 24 h (A and C) or 48 h (B and D) in culture with the PAMPs ultrapure LPS, LTA, PGN, or Pam3CSK4. Granulosa cells were treated with 10-fold increasing doses of each PAMP from 100 pg/ml up to 10 μg/ml (ranging *left to right* and represented by *triangles*). Data are presented as mean + SEM from four independent experiments. *, $P < 0.05$ compared with untreated controls; analysis by ANOVA followed by Dunnett's pair-wise *post hoc* tests.

Granulosa cells produce an inflammatory response to PAMPs

Granulosa cells were challenged with a range of bacterial PAMPs to determine their ability to signal through TLR and initiate an inflammatory response, as determined by the accumulation of the proinflammatory cytokine IL-6 and the chemokine IL-8. Initially, granulosa cells were cultured in either serum-free medium (25) or medium containing 10% FCS. Granulosa cells had little or no response to PAMPs in serum-free conditions (Supplemental Fig. 1). All further cultures were performed with serum and granulosa cells incubated for 24 h in the presence of LPS (TLR4 ligand), LTA (TLR2 ligand), PGN (TLR2 ligand), or Pam3CSK4 (TLR1 and TLR2 synthetic ligand) accumulated IL-6 and IL-8 in supernatants in a dose-dependent manner ($P < 0.05$) (Fig. 2, A and C). The accumulation of IL-6 and IL-8 was further increased after 48 h of culture with each PAMP compared with 24 h ($P < 0.05$) (Fig. 2, B and D). The remaining experiments used 1 μg/ml LPS, because granulosa cells had the greatest sensitivity to LPS (Fig. 2), the follicular fluid of cattle contains LPS (2), and similar concentrations of LPS are used to study immune cell activation (20).

Granulosa cells respond acutely to LPS

To determine the acute response of granulosa cells to LPS, we used Western blotting, real-time RT-PCR, and live cell imaging to evaluate initiation of the signaling cascade, *IL6* and *IL8* mRNA expression, and p65-NFκB nuclear translocation, respectively.

Granulosa cells before LPS exposure showed little or no initial phosphorylation of p38 or ERK1/2, but after a 30-min treatment with 1 μg/ml LPS, there was phosphorylation of both ERK1/2 and p38, which was maintained up to 180 min (Fig. 3A).

Granulosa cells were challenged with 1 μg/ml LPS for 0, 30, 60, 90, or 180 min to determine *IL6* and *IL8* mRNA expression. The *IL6* mRNA was increased by 2.1-fold in cells after only a 30-min treatment compared with untreated controls and remained 2.5-fold elevated at 180 min ($P < 0.05$) (Fig. 3B); *IL8* mRNA was elevated 2.6-fold after 30 min compared with untreated controls and remained elevated 5.3-fold at 180 min ($P < 0.05$) (Fig. 3C).

Because nuclear translocation of NFκB is part of the TLR signaling pathway (12, 13), cells transfected with IκBα-EGFP and p65-dsRed were monitored for 3 h using live cell confocal microscopy (32). Treatment of cells with 20 ng/ml TNFα as a positive control showed both degradation of IκBα-EGFP (Fig. 4, A and B) and nuclear translocation of p65-dsRed (Fig. 4, C and D) after 90 min in approximately 20% of analyzed cells. Untreated and LPS-treated cells showed no breakdown of IκBα-EGFP (Fig. 4, E and F) or nuclear translocation of p65-dsRed (Fig. 4, G and H) over 90 min, and there was no change when visualized for a further 90 min.

LPS-induced inflammatory response of granulosa cells is attenuated by siRNA-TLR4

The recognition of LPS by cells of the immune system is mediated through TLR4 (19, 20); thus, *TLR4*-targeted siRNA was used to determine whether LPS-induced inflammatory responses by bovine granulosa cells are also dependent on TLR4. The siRNA reduced *TLR4* expression in granulosa cells either untreated or treated with 1 μg/ml LPS ($P < 0.05$) (Fig. 5A). Furthermore, siRNA tar-
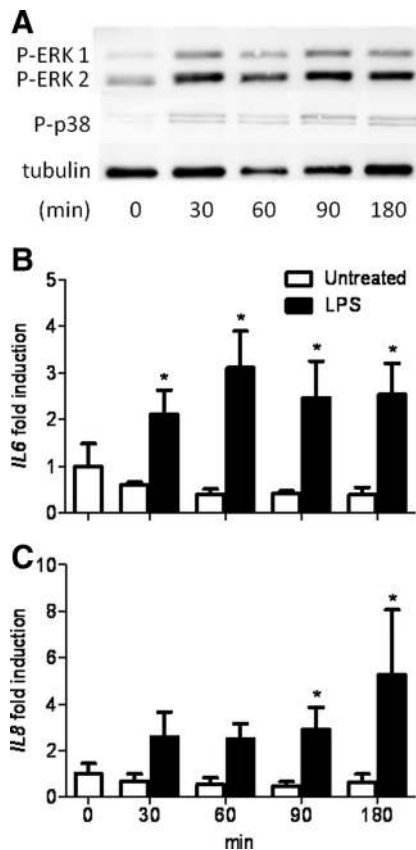
FIG. 3. LPS induced phosphorylation of ERK and p38, and *IL6* and *IL8* gene expression in granulosa cells in a time-dependent manner. A, Granulosa cells were treated with 1 μg/ml ultrapure LPS and cultured for 30, 60, 90, or 180 min before Western blot analysis. Bands are shown corresponding to diphosphorylated ERK1/2, phosphorylated p38 (Thr180/Tyr182), and tubulin as a loading control. Image is representative of three independent experiments. Granulosa cells were cultured in control medium (□) or medium containing 1 μg/ml ultrapure LPS (■) for 30, 60, 90, or 180 min, and the *IL6* (B) and *IL8* (C) gene expression was measured using real-time RT-PCR. Data are represented as mean + SEM fold induction compared with control cells at time 0 from four independent experiments. *, $P < 0.05$ compared with untreated control within time points; analysis by nonparametric Mann-Whitney *U* test.

geting of TLR4 reduced the accumulation of IL-6 in response to LPS ($P < 0.05$) (Fig. 5B).

Exogenous gonadotropins alter innate immune responses to LPS

Granulosa cells from the growing pool of follicles (4–8 mm) are FSH responsive and LH receptor negative (24). Previously, *Tlr4*, *Cd14*, and *Myd88* mRNA expression was shown to increase in murine COCs due to FSH exposure *in vitro* (14). Here, exogenous recombinant bovine-FSH was used to assess its role in mediating the inflammatory response in granulosa cells. To determine whether FSH *per se* had an effect on innate immunity, as in lower organisms (34), we investigated whether FSH alone induced changes in phosphorylation of p38 and
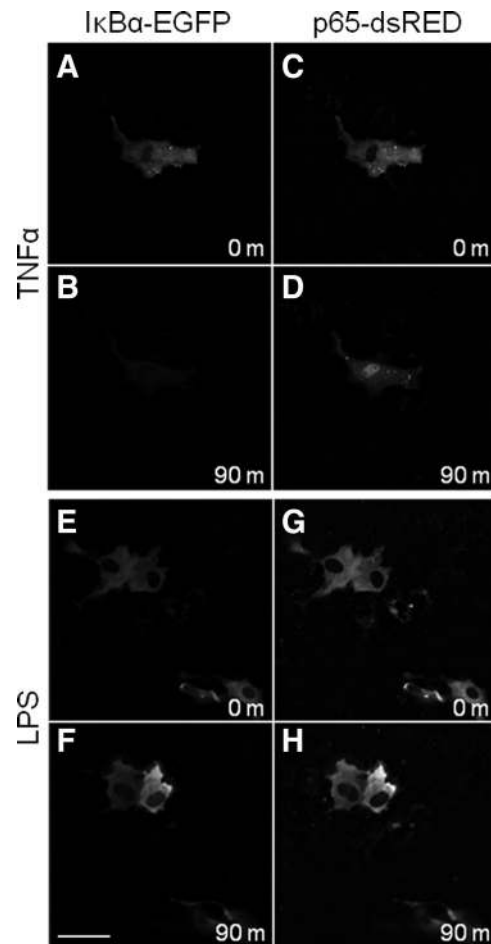

FIG. 4. Nuclear translocation of p65 or IκBα degradation in granulosa cells was induced by TNFα but not LPS. Granulosa cells were cultured in glass bottom Petri dishes and transfected with IκBα-EGFP (A and B and E and F) and p65-dsRed (C, D, G and H). Live cell confocal microscopy under culture conditions was performed to track degradation of IκBα-EGFP and nuclear translocation of p65-dsRed. Cells were treated with 20 ng/ml TNFα (A–D) as a positive control or 1 μg/ml ultrapure LPS (E–H). *Scale bar*, 50 μm.

ERK1/2, *IL6* and *IL8* mRNA, and accumulation of IL-6 and IL-8. In the absence of LPS, FSH alone did not increase phosphorylation of p38 or ERK1/2 (data not shown), or *IL6* or *IL8* mRNA after 180 min (71 and 21% of control, respectively). Additionally, the accumulation of IL-6 or IL-8 was unchanged when treated with FSH alone (1.0- and 1.8-fold, respectively).

To explore interactions between gonadotropins and LPS, granulosa cells were either pretreated with FSH for 6 h before LPS exposure or treated with FSH for the duration of the LPS exposure. In the absence of gonadotropins, as expected, there was a dose-dependent accumulation of IL-6 and IL-8 after treatment of granulosa cells with LPS for 24 h ($P < 0.05$) (Fig. 6, A and C) or 48 h ($P < 0.05$) (Fig. 6, B and D). Pretreatment or continuous treatment of granulosa cells with FSH had no further effect on the IL-6 accumulation after 24 h of LPS exposure (Fig.
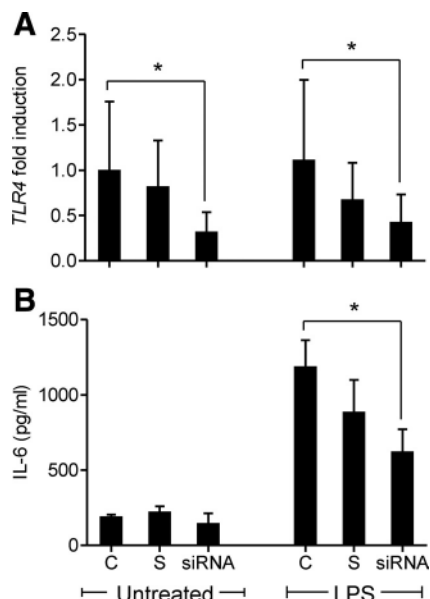
FIG. 5. siRNA targeting of *TLR4* in granulosa cells reduced *TLR4* gene expression and the accumulation of IL-6 in response to LPS. Granulosa cells were cultured for 24 h with lipofectamine [control (C)], scramble-siRNA (S), or a specific *TLR4*-targeted siRNA (siRNA) before treatment with 1 μg/ml ultrapure LPS for an additional 24 h. A, Real-time RT-PCR was used to evaluate the knockdown of *TLR4* mRNA with expression presented as fold-change relative to untreated controls. B, IL-6 accumulation was measured in cell-free supernatants by ELISA after a 24-h treatment with 1 μg/ml ultrapure LPS. Data are presented as mean + SEM from four independent experiments. *, $P < 0.05$ compared with controls within treatment group. Real-time RT-PCR data were analyzed using a nonparametric Mann-Whitney *U* test, ELISA data were analyzed using ANOVA followed by a Bonferonni *post hoc* test.

6A). However, after a 48-h exposure to LPS, pretreatment or continuous treatment with FSH increased accumulation of IL-6 compared with cells with no FSH treatment ($P < 0.05$) (Fig. 6B). The accumulation of IL-8 after a 24- or 48-h exposure to LPS was significantly increased when granulosa cells were pretreated or continuously treated with FSH compared with those with no FSH treatment ($P < 0.05$) (Fig. 6, C and D). There was no significant interaction between FSH treatment and LPS dose in each experiment.

COC IVM is perturbed by LPS exposure

Because LPS stimulated an inflammatory response in granulosa cells, and in the mouse IL-6 is critical for COC expansion and can bypass amphiregulin-dependent expansion of the COC (35), we posited that this could change the follicular environment for developing oocytes. Therefore, we performed IVM of COCs using defined medium with the addition of exogenous LPS for 24 h to determine perturbations in IL-6 production (216 COCs used in four independent experiments), COC expansion (422 COCs used across eight independent experiments), and

meiotic maturation (290 COCs used across three independent experiments). Treatment of COCs with 1 or 10 μg/ml LPS induced 76 and 58% increase in IL-6 accumulation compared with untreated controls, respectively ($P < 0.05$) (Fig. 7A). Examination of the expansion of COCs revealed that in the absence of FSH, 10 μg/ml LPS significantly increased the rates of cumulus expansion compared with untreated controls (0.0 *vs*. 24.0%, $P < 0.05$) (Fig. 7B).

After 24 h of IVM, oocytes were subjected to confocal microscopy to determine their meiotic status. Morphologically normal oocytes containing bipolar spindles and aligned chromosomes along the meta-phase plate were deemed normal MII oocytes (Fig. 8A). Oocytes that failed to reach the M-phase of meiosis II, or those with perturbed meiotic structures (aberrant spindles, chromosomal ejection, or parthenogenic activation), were deemed to have failed meiosis (Fig. 8B). In all, 290 COCs were assessed in eight treatment groups. Control IVM consisting of basal medium with no exogenous gonadotropins resulted in a meiotic failure rate of 14.0%, with the addition of 10 μg/ml LPS the meiotic failure rate significantly increased to 34.4% ($P < 0.05$). IVM in the presence of both FSH and LH resulted in a 14.3% meiotic failure rate, but the addition of 10 μg/ml LPS again increased the meiotic failure rate to 35.7% ($P < 0.05$) (Fig. 8C).

Discussion

Reproductive tract infection in dairy cattle is common after parturition and leads to endocrine dysfunction and ultimately infertility (5, 6). The Gram-negative bacteria, *E. coli*, is the most prevalent pathogen detected in the uterus post partum (2, 9). Although infection of the ovary itself is rare, animals with postpartum uterine infection have concentrated LPS in the follicular fluid of growing follicles (2). Previously, it has been demonstrated that in the absence of LPS, TLR4 may play a role in ovulation in the mouse by using endogenous ligands (21). In contrast, here, we explore the role of granulosa cells in a pathological context and hypothesize that LPS from Gram-negative bacteria stimulates an inflammatory response by ovarian granulosa cells that is mediated by TLR4. Absence of immune cells within the ovarian follicle or granulosa cell cultures in the present study further highlight potential roles for granulosa cells in innate immunity. In the present study, the bacterial PAMPs LPS, LTA, PGN, and Pam3CSK4 initiated an inflammatory response by granulosa cells in a dose-dependent manner with accumulation of the inflammatory cytokine IL-6 and the chemokine IL-8. Using LPS as the prototypical PAMP, granulosa cells
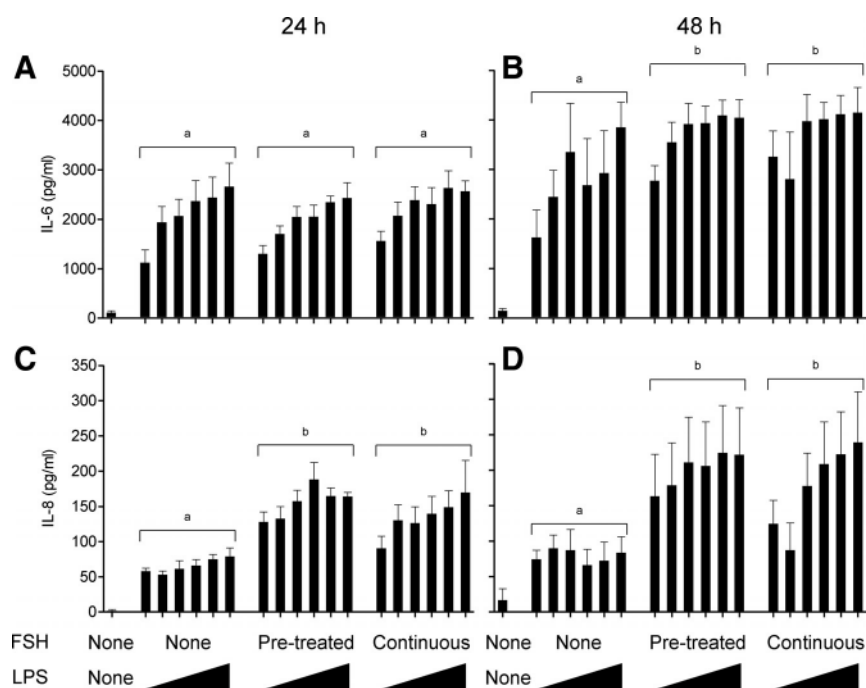
FIG. 6. Exogenous FSH increases accumulation of IL-6 and IL-8 in response to LPS by granulosa cells. Accumulation of IL-6 (A and B) and IL-8 (C and D) measured by ELISA after 24 h (A and C) or 48 h (B and D) in culture after challenge of granulosa cells with ultrapure LPS. Granulosa cells were either preincubated with 2.5 μg/ml FSH for 6 h before LPS exposure or treated with 2.5 μg/ml FSH for the duration of the LPS exposure. Granulosa cells were challenged with 10-fold increasing doses of ultrapure LPS between 100 pg/ml and 10 μg/ml (ranging *left to right* and represented by *triangles*). Data are presented as mean + SEM from four independent experiments. *Different superscripts* represent statistically significant differences between FSH treatment groups ($P < 0.05$); analysis by ANOVA followed by Bonferonni *post hoc* test.

licles (37), these data present the intriguing possibility that granulosa cells could act as immune sensors. Here, we demonstrated granulosa cell functional responsiveness to PAMPs for TLR1, TLR2, and TLR4. Functional TLR1 and TLR2 are relevant, because Gram-positive bacteria, which possess their ligands, commonly causes metritis (1).

The proinflammatory cytokine IL-6 is a potent regulator of early inflammation and is commonly increased in response to LPS (36). However, IL-6 also has a critical role in the ovary. Temporal regulation of IL-6 is important in coordinating meiotic maturation, COC expansion, ovulation, and corpus luteum formation (35, 38, 39). The chemokine IL-8 is critical for the recruitment of leukocytes into the site of infection and, similar to IL-6, is up-regulated in response to LPS via the TLR4 pathway (36). The role of IL-8 in the ovary is less well characterized, with a suggested role in the recruitment of leukocytes after ovulation, which then aid in the formation and function of the corpus luteum (40, 41). We surmise that in granulosa cells, altered expression of IL-8 in response to LPS is involved in aberrant inflammation in the ovary, whereas altered IL-6 expression may have a direct impact on the oocyte, negatively affecting meiotic maturation.

Binding of LPS to TLR4 on immune cells activates the MAPK and NFκB pathways to increase expression of inflammatory mediators (13, 42). In the present study, initial activation of both p38 and ERK was visualized in response to LPS after 30 min of treatment. Also, silencing of TLR4 by specific siRNA targeting reduced LPS initiated inflammation, providing further evidence that TLR4 signaling is important in a pathophysiological response to LPS in bovine granulosa cells. It is also interesting to note that bovine granulosa cell mRNA expression of *TLR4* was not increased in response to LPS (43).

Gonadotropins are an essential element in successful development and function of the ovarian follicle (16, 17). Granulosa cells used in the present study were selected from the 4- to 8-mm pool of growing follicles, which provided a homogenous population of granulosa cells with limited apoptotic cells, similar to the environment of the developing oocyte. These granulosa cells are known to be FSH receptor positive and LH receptor negative (24). In
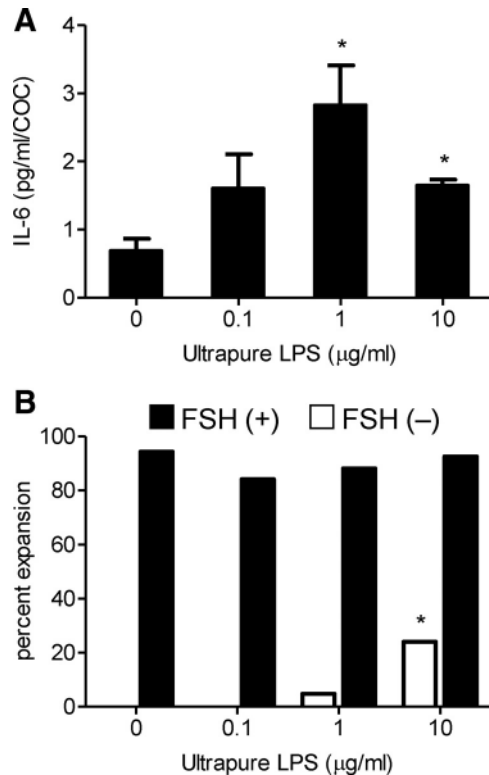
initiated phosphorylation of the downstream mediators ERK and p38 and acute up-regulation of *IL6* and *IL8* mRNA. Accumulation of IL-6 and IL-8 was evident with low doses of LPS, akin to the responsiveness of immune cells (36). This response to LPS was reduced by siRNA targeting TLR4 of granulosa cells. Interestingly, the addition of FSH into the culture system increased granulosa cell sensitivity and responsiveness to LPS. Furthermore COCs accumulated IL-6 in response to LPS and underwent aberrant expansion in the absence of exogenous gonadotropins. Finally, IVM of oocytes was perturbed by the presence of exogenous LPS, resulting in their failure to complete meiosis. Taken together, these data support the concept that granulosa cells have roles in innate immunity and provide mechanisms for perturbation of fertility by bacterial infections.

Bovine granulosa cells express the molecular machinery (*TLR4*, *CD14*, and *MD2*) important for initiating the innate immune response to Gram-negative pathogens (2). LPS accumulates in follicular fluid of animals with metritis, and microbial colonization of human follicular fluid occurs in *in vitro* fertilization patients (11). Taken together with the absence of immune cells from ovarian fol-

FIG. 7. LPS induces IL-6 accumulation and expansion in *in vitro* matured COCs. A, IL-6 accumulation in cell-free supernatants was measured from groups of 10–20 COCs treated with 0, 0.1, 1, or 10 μg/ml LPS in the presence of 2.5 μg/ml FSH. IL-6 concentrations are presented as mean pg/ml per COC + SEM from a total of 216 across four independent experiments. *, $P < 0.05$ compared with untreated controls; analysis by ANOVA followed by Dunnett's *post hoc* test. B, COC expansion rates were recorded from groups of 10–20 COCs, after treatment with 0, 0.1, 1, or 10 μg/ml LPS in the absence (□) or presence (■) of 2.5 μg/ml FSH. COC expansion data are presented as percentages from a total of 422 COCs across eight independent experiment. *, $P < 0.05$ compared with untreated COCs within FSH treatment group using a χ^2 test.

FIG. 8. A, LPS induces meiotic failure in *in vitro* matured COCs. Meiotic progression was assessed in 290 COCs by confocal microscopy. Oocytes at the M-phase of meiosis with a normal bipolar spindle and aligned chromosomes were deemed to have completed meiosis. B, Any oocyte not to reach the M-phase of meiosis II or those which had significantly perturbed meiotic structures such as aberrant spindles, chromosomal ejection, parthenogenic activation, or germinal vesicle breakdown failure were deemed to have failed meiosis. Representative confocal micrographs of COCs show DNA (*blue*), tubulin (*green*), and F-actin (*red*). C, Meiotic failure rates of COCs cultured in the defined IVM media containing LPS, FSH, and/or LH (as indicated) for 24 h are presented as *percentages*. *Different superscripts* represent statistical significance after a χ^2 test; $P < 0.05$. PB, Polar body. *Scale bar*, 50 μm.

mice, the treatment of COCs with FSH increased mRNA of *Tlr4*, *Myd88*, and *Cd14* but not in granulosa cells (14). In *Caenorhabditis elegans*, the FSH receptor plays an integral role in the innate immune response to bacterial PAMPs by eliciting functional inflammatory responses to pathogens (34). To examine the impact of FSH in bovine granulosa cell immunity here cells were treated with FSH before or during challenge with LPS. The addition of FSH to granulosa cells increased the accumulation of IL-6 and IL-8 in response to LPS and in addition increased their sensitivity to LPS. In the present study, in the absence of LPS, the addition of FSH alone to granulosa cell cultures did not increase p38 or ERK1/2 phosphorylation, *IL6* or *IL8* mRNA production, or IL-6 or IL-8 accumulation in supernatants. This varies from studies in the mouse where TLR4 and FSH-receptor both act in part via ERK1/2 signaling pathways (44), increasing IL-6 production in re-
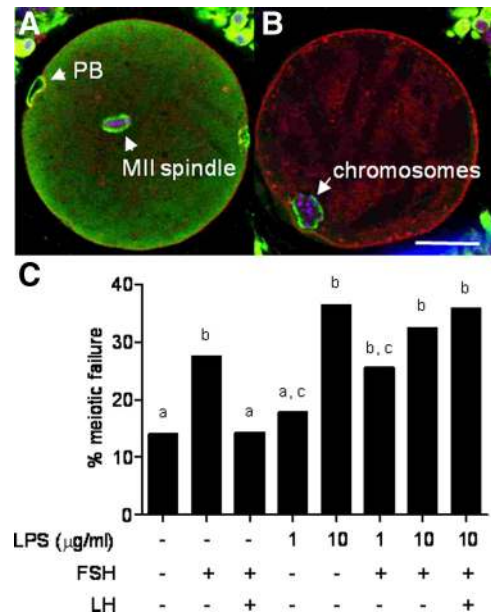
sponse to FSH (14). Alternatively, in bovine granulosa cells, FSH may signal via the AKT or phosphatidylinositol 3-kinase pathways to initiate endocrine changes important in their function (45, 46).

The process of COC expansion is critical for ovulation and fertilization and is indicative of oocyte quality (47). Expansion of the COC requires intrinsic signaling molecules expressed in a temporal manner in response to the LH surge and their downstream products, such as IL-6 (35). We investigated the ability of LPS to induce COC expansion and IL-6 production in the absence of gonadotropins. Although LPS induced IL-6 accumulation during IVM of bovine COCs, high doses of LPS were required to induce gonadotropin-independent expansion above the levels detected in follicular fluid of infected cattle. We suggest that in the cow, LPS may induce the increased expression of cumulus derived factors needed for COC expansion, including IL-6 (35).

Cattle suffering uterine or mammary infection have delayed conception, suggesting failure to conceive as a result of uterine and/or ovarian dysfunction (6, 48). Meiotic maturation is an essential process in readying both the

nuclear and cytoplasmic compartments of the oocyte for ovulation, fertilization, and the first cleavage events of the new zygote. In cattle, meiotic maturation from the germinal vesicle stage to MII takes approximately 60 d, whereas *in vitro*, the process can be accelerated with the use of exogenous gonadotropins and completed in 24 h. Here, maturation to the MII stage of meiosis was perturbed by the presence of LPS with no protective effect of exogenous gonadotropins. Production of IL-6 within the ovarian follicle seems to be temporally modulated with elevated IL-6 in follicles containing mature oocytes compared with those containing immature oocytes (38). In the rat, LPS administration leads to increased follicular atresia and granulosa cell apoptosis (49), processes closely linked to increased IL-8 expression (50). Oocytes collected from dogs suffering uterine infection have shown very low IVM rates (51). In addition, women suffering endometriosis have poor oocyte quality and elevated IL-6 in the circulation (52) and increased IL-6 production by their granulosa cells (53). It would appear that although IL-6 expression within the ovary is important for oocyte development, appropriate temporal expression is critical. Thus, altered oocyte competence due to LPS exposure, increased by FSH, may be a protective mechanism developed by the ovary to prevent the maturation and fertilization of suboptimal quality oocytes, whereas elevated IL-8 may increase follicle atresia.

Because bacterial infection of the ovary itself is a rare event, inhibition of key downstream regulators of the TLR4 signaling pathway may provide suitable targets for blocking the aberrant inflammatory response to LPS within follicles. Here, p65 nuclear translocation and IκB degradation required for classical NFκB signaling (54) was absent. Surprisingly, the kinetics of p65 nuclear translocation and IκB degradation in TNFα-treated granulosa cells was reduced by an order of magnitude compared with immune cells (32). It is important to note the number of components within the NFκB pathway, some of which can increase NFκB responsive genes in the absence of p65 and may explain the ability of granulosa cells to initiate LPS-induced inflammatory responses in granulosa cells without p65 nuclear translocation (55).

In conclusion, bovine granulosa cells initiated an inflammatory response to PAMPs and the response to LPS was via the TLR4 pathway. Intriguingly, there may be interactions between endocrine and immune pathways, because the granulosa cells were more sensitive to LPS in the presence of FSH. The impact of LPS is not limited to granulosa cells, because COCs underwent aberrant cumulus expansion and increased meiotic failure *in vitro*.

The present studies support the concept that bovine granulosa cells have roles in innate immunity that are linked to fertility.

References

1. **Sheldon IM, Noakes DE, Rycroft AN, Pfeiffer DU, Dobson H** 2002 Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. Reproduction 123:837–845
2. **Herath S, Williams EJ, Lilly ST, Gilbert RO, Dobson H, Bryant CE, Sheldon IM** 2007 Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. Reproduction 134:683–693
3. **Williams EJ, Sibley K, Miller AN, Lane EA, Fishwick J, Nash DM, Herath S, England GC, Dobson H, Sheldon IM** 2008 The effect of *Escherichia coli* lipopolysaccharide and tumour necrosis factor α on ovarian function. Am J Reprod Immunol 60:462–473
4. **Lavon Y, Leitner G, Klipper E, Moallem U, Meidan R, Wolfenson D** 2011 Subclinical, chronic intramammary infection lowers steroid concentrations and gene expression in bovine preovulatory follicles. Domest Anim Endocrinol 40:98–109
5. **Dohmen MJ, Joop K, Sturk A, Bols PE, Lohuis JA** 2000 Relationship between intra-uterine bacterial contamination, endotoxin levels and the development of endometritis in postpartum cows with dystocia or retained placenta. Theriogenology 54:1019–1032
6. **Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth HJ** 2009 Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. Biol Reprod 81:1025–1032
7. **Lavon Y, Ezra E, Leitner G, Wolfenson D** 2011 Association of conception rate with pattern and level of somatic cell count elevation relative to time of insemination in dairy cows. J Dairy Sci 94:4538–4545
8. **Hertl JA, Gröhn YT, Leach JD, Bar D, Bennett GJ, González RN, Rauch BJ, Welcome FL, Tauer LW, Schukken YH** 2010 Effects of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on the probability of conception in New York State Holstein dairy cows. J Dairy Sci 93:1551–1560
9. **Sheldon IM, Rycroft AN, Dogan B, Craven M, Bromfield JJ, Chandler A, Roberts MH, Price SB, Gilbert RO, Simpson KW** 2010 Specific strains of *Escherichia coli* are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. PLoS One 5:e9192
10. **Ross JD** 2002 An update on pelvic inflammatory disease. Sex Transm Infect 78:18–19
11. **Pelzer ES, Allan JA, Cunningham K, Mengersen K, Allan JM, Launchbury T, Beagley K, Knox CL** 2011 Microbial colonization of

follicular fluid: alterations in cytokine expression and adverse assisted reproduction technology outcomes. Hum Reprod 26:1799–1812

12. **Beutler B** 2004 Inferences, questions and possibilities in toll-like receptor signalling. Nature 430:257–263

13. **Takeuchi O, Akira S** 2010 Pattern recognition receptors and inflammation. Cell 140:805–820

14. **Shimada M, Hernandez-Gonzalez I, Gonzalez-Robanya I, Richards JS** 2006 Induced expression of pattern recognition receptors in cumulus oocyte complexes: novel evidence for innate immune-like functions during ovulation. Mol Endocrinol 20:3228–3239

15. **Spanel-Borowski K, Rahner P, Ricken AM** 1997 Immunolocalization of CD18-positive cells in the bovine ovary. J Reprod Fertil 111:197–205

16. **Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ** 2001 Cellular basis for paracrine regulation of ovarian follicle development. Reproduction 121:647–653

17. **Matzuk MM, Burns KH, Viveiros MM, Eppig JJ** 2002 Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296:2178–2180

18. **Eppig JJ** 1996 Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reprod Fertil Dev 8:485–489

19. **Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B** 1998 Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282:2085–2088

20. **Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S** 1999 Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol 162:3749–3752

21. **Shimada M, Yanai Y, Okazaki T, Noma N, Kawashima I, Mori T, Richards JS** 2008 Hyaluronan fragments generated by sperm-secreted hyaluronidase stimulate cytokine/chemokine production via the TLR2 and TLR4 pathway in cumulus cells of ovulated COCs, which may enhance fertilization. Development 135:2001–2011

22. **Spanel-Borowski K** 2011 Ovulation as danger signaling event of innate immunity. Mol Cell Endocrinol 333:1–7

23. **Espey LL** 1980 Ovulation as an inflammatory reaction—a hypothesis. Biol Reprod 22:73–106

24. **Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RS** 1995 Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. Biol Reprod 53:951–957

25. **Gutiérrez CG, Campbell BK, Webb R** 1997 Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics. Biol Reprod 56:608–616

26. **Fukui Y, Ono H** 1989 Effects of sera, hormones and granulosa cells added to culture medium for in-vitro maturation, fertilization, cleavage and development of bovine oocytes. J Reprod Fertil 86:501–506

27. **Rose TA, Bavister BD** 1992 Effect of oocyte maturation medium on in vitro development of in vitro fertilized bovine embryos. Mol Reprod Dev 31:72–77

28. **Combelles CM, Cekleniak NA, Racowsky C, Albertini DF** 2002 Assessment of nuclear and cytoplasmic maturation in in-vitro matured human oocytes. Hum Reprod 17:1006–1016

29. **Bromfield JJ, Coticchio G, Hutt K, Sciajno R, Borini A, Albertini DF** 2009 Meiotic spindle dynamics in human oocytes following slow-cooling cryopreservation. Hum Reprod 24:2114–2123

30. **Rico C, Médigue C, Fabre S, Jarrier P, Bontoux M, Clément F, Monniaux D** 2011 Regulation of anti-Mullerian hormone production in the cow: a multiscale study at endocrine, ovarian, follicular, and granulosa cell levels. Biol Reprod 84:560–571

31. **Nelson G, Paraoan L, Spiller DG, Wilde GJ, Browne MA, Djali PK,** Unitt JF, Sullivan E, Floettmann E, White MR 2002 Multi-parameter analysis of the kinetics of NF-κB signalling and transcription in single living cells. J Cell Sci 115:1137–1148

32. **Sée V, Rajala NK, Spiller DG, White MR** 2004 Calcium-dependent regulation of the cell cycle via a novel MAPK-NF-κB pathway in Swiss 3T3 cells. J Cell Biol 166:661–672

33. **Rinaldi M, Ceciliani F, Lecchi C, Moroni P, Bannerman DD** 2008 Differential effects of α1-acid glycoprotein on bovine neutrophil respiratory burst activity and IL-8 production. Vet Immunol Immunopathol 126:199–210

34. **Powell JR, Kim DH, Ausubel FM** 2009 The G protein-coupled receptor FSHR-1 is required for the *Caenorhabditis elegans* innate immune response. Proc Natl Acad Sci USA 106:2782–2787

35. **Liu Z, de Matos DG, Fan HY, Shimada M, Palmer S, Richards JS** 2009 Interleukin-6: an autocrine regulator of the mouse cumulus cell-oocyte complex expansion process. Endocrinology 150:3360–3368

36. **Dentener MA, Bazil V, Von Asmuth EJ, Ceska M, Buurman WA** 1993 Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-α, IL-6 and IL-8 release by human monocytes and alveolar macrophages. J Immunol 150:2885–2891

37. **Brännström M, Mayrhofer G, Robertson SA** 1993 Localization of leukocyte subsets in the rat ovary during the periovulatory period. Biol Reprod 48:277–286

38. **Kawasaki F, Kawano Y, Kosay Hasan Z, Narahara H, Miyakawa I** 2003 The clinical role of interleukin-6 and interleukin-6 soluble receptor in human follicular fluids. Clin Exp Med 3:27–31

39. **Richards JS, Liu Z, Shimada M** 2008 Immune-like mechanisms in ovulation. Trends Endocrinol Metab 19:191–196

40. **Arici A, Oral E, Bukulmez O, Buradagunta S, Engin O, Olive DL** 1996 Interleukin-8 expression and modulation in human preovulatory follicles and ovarian cells. Endocrinology 137:3762–3769

41. **Murayama C, Kaji A, Miyauchi K, Matsui M, Miyamoto A, Shimizu T** 2010 Effect of VEGF (vascular endothelial growth factor) on expression of IL-8 (interleukin-8), IL-1β and their receptors in bovine theca cells. Cell Biol Int 34:531–536

42. **Medzhitov R, Preston-Hurlburt P, Janeway Jr CA** 1997 A human homologue of the *Drosophila* toll protein signals activation of adaptive immunity. Nature 388:394–397

43. **Muzio M, Bosisio D, Polentarutti N, D'amico G, Stoppacciaro A, Mancinelli R, van't Veer C, Penton-Rol G, Ruco LP, Allavena P, Mantovani A** 2000 Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J Immunol 164:5998–6004

44. **Wayne CM, Fan HY, Cheng X, Richards JS** 2007 Follicle-stimulating hormone induces multiple signaling cascades: evidence that activation of Rous sarcoma oncogene, RAS, and the epidermal growth factor receptor are critical for granulosa cell differentiation. Mol Endocrinol 21:1940–1957

45. **Das S, Maizels ET, DeManno D, St Clair E, Adam SA, Hunzicker-Dunn M** 1996 A stimulatory role of cyclic adenosine 3′,5′-monophosphate in follicle-stimulating hormone-activated mitogen-activated protein kinase signaling pathway in rat ovarian granulosa cells. Endocrinology 137:967–974

46. **Mani AM, Fenwick MA, Cheng Z, Sharma MK, Singh D, Wathes DC** 2010 IGF1 induces up-regulation of steroidogenic and apoptotic regulatory genes via activation of phosphatidylinositol-dependent kinase/AKT in bovine granulosa cells. Reproduction 139:139–151

47. **Larsen WJ, Chen L, Powers R, Zhang H, Russell PT, Chambers C, Hess K, Flick R** 1996 Cumulus expansion initiates physical and developmental autonomy of the oocyte. Zygote 4:335–341

48. **Williams EJ, Fischer DP, Noakes DE, England GC, Rycroft A, Dobson H, Sheldon IM** 2007 The relationship between uterine pathogen growth density and ovarian function in the postpartum dairy cow. Theriogenology 68:549–559

49. **Besnard N, Horne EA, Whitehead SA** 2001 Prolactin and lipopolysaccharide treatment increased apoptosis and atresia in rat ovarian follicles. Acta Physiol Scand 172:17–25

50. **Połeć A, Tanbo T, Fedorcsák P** 2009 Cellular interaction regulates interleukin-8 secretion by granulosa-lutein cells and monocytes/macrophages. Am J Reprod Immunol 61:85–94

51. **Hishinuma M, Minami S, Okamoto Y, Miyatake K, Sekine J** 2004 Recovery, morphological quality, and in vitro maturation of follicular oocytes from bitches with pyometra. Theriogenology 62:1652–1662

52. **Martínez S, Garrido N, Coperias JL, Pardo F, Desco J, García-Velasco JA, Simón C, Pellicer A** 2007 Serum interleukin-6 levels are elevated in women with minimal-mild endometriosis. Hum Reprod 22:836–842

53. **Carlberg M, Nejaty J, Fröysa B, Guan Y, Söder O, Bergqvist A** 2000 Elevated expression of tumour necrosis factor α in cultured granulosa cells from women with endometriosis. Hum Reprod 15:1250–1255

54. **Henkel T, Machleidt T, Alkalay I, Krönke M, Ben-Neriah Y, Baeuerle PA** 1993 Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature 365:182–185

55. **Kim JM, Oh YK, Kim YJ, Youn J, Ahn MJ** 2004 *Escherichia coli* up-regulates proinflammatory cytokine expression in granulocyte/macrophage lineages of CD34 stem cells via p50 homodimeric NF-κB. Clin Exp Immunol 137:341–350