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Lipopolysaccharide Reduces the Primordial Follicle Pool in the Bovine Ovarian Cortex Ex Vivo and in the Murine Ovary In Vivo[1]

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ABSTRACT

Infections of the uterus or mammary gland with Gram-negative bacteria cause infertility in cattle, not only during disease but also for some time afterward. Even though these infections are in organs distant from the ovary, metritis and mastitis perturb antral follicle development and function in vivo. Although granulosa cells from antral follicles express toll-like receptor 4 (TLR4), and detect and mount an inflammatory response to lipopolysaccharide (LPS) from Gram-negative bacteria, it is not known whether LPS impacts preantral follicle development. The present study tested the hypothesis that LPS perturbs the development of primordial ovarian follicles. Exposure of bovine ovarian cortex ex vivo to LPS reduced the primordial follicle pool associated with increased primordial follicle activation. Ovarian cortex culture supernatants accumulated the inflammatory mediators IL-1beta, IL-6, and IL-8 in an LPS concentration-dependent manner. In addition, LPS exposure modulated key intracellular regulators of follicle activation with loss of the primordial follicle PTEN and cytoplasmic translocation of FOXO3. Acute exposure of mice in vivo to LPS also reduced the primordial follicle pool associated with increased follicle atresia. The increased follicle atresia was TLR4-dependent as *Tlr4*-deficient mice were insensitive to LPS-mediated follicle atresia. However, LPS did not affect the diameter of individually cultured bovine secondary follicles or their enclosed oocytes. In conclusion, LPS reduced the primordial ovarian follicle pool in the bovine ovarian cortex ex vivo and in the murine ovary in vivo. These observations provide an insight into how bacterial infections distant from the ovary have long term effects on fertility.

immunity, infection, inflammation, lipopolysaccharide, ovarian cortex, ovarian follicle, ovary, toll-like receptor

INTRODUCTION

Infections of the bovine uterus and mammary gland with Gram-negative bacteria commonly cause metritis and mastitis, respectively. Uterine infections following parturition cause metritis in 40% of animals, and the resulting decreased milk yields, infertility, and animal culling for failure to conceive cost the U.S. dairy industry $650 million/year [1]. The incidence of mastitis is at least as common as metritis, and mastitis is also associated with reduced fertility [2]. Metritis and mastitis perturbation result in several aspects of ovarian function, including retarded follicular growth, reduced circulating and intrafollicular estradiol concentrations, altered duration of luteal phases, and disruption of ovarian cyclic activity [3–6]. However, infections of the uterus and mammary gland not only cause infertility at the time of infection but reduced fecundity also persists even after resolution of disease [1, 7]. An intriguing question about biological mechanisms is how these Gram-negative bacterial infections in tissues distant from the ovary can impact ovarian function?

Escherichia coli is the most common Gram-negative pathogen causing metritis and mastitis, and much of the tissue pathology is associated with the bacterial endotoxin lipopolysaccharide (LPS). One link between bacterial infection and ovarian dysfunction is the accumulation of LPS in the follicular fluid of animals with metritis in vivo [4]. Estradiol is reduced in granulosa cells cultured with LPS [5], while animals with mastitis have altered granulosa cell gene expression and lower follicular estradiol [6]. The innate immune system relies on pattern recognition receptors on host mammalian cells to detect microbe- or pathogen-associated molecular patterns, including LPS [8]. Specifically, LPS is bound by toll-like receptor 4 (TLR4) in concert with the coreceptors CD14 and MD-2, which stimulates cellular responses characterized by the production of inflammatory mediators, typically interleukin-1β (IL-1β), IL-6, and IL-8 [9]. The role of TLR4 is often studied using cells of the immune system, particularly in mice because of the availability of *Tlr4*-deficient ($Tlr4^{-/-}$) animals [10]. However, TLR4 is also expressed and functional in cells of the gastrointestinal, respiratory, and female reproductive systems [11–13]. Recently we have shown that LPS induces a TLR4-dependent inflammatory response by bovine granulosa cells collected from antral follicles and that LPS perturbs oocyte development [14]. What is less clear is whether LPS affects the primordial follicle or the preantral stages of follicle development.

The mammalian ovary contains a finite number of oocytes encapsulated as quiescent primordial follicles [15]. Development of a competent oocyte and subsequent fertility depend on the coordinated activation and development of primordial follicles along with close regulation of granulosa cell proliferation and intercommunication between the oocyte and granulosa cells [16]. Key cellular determinants of primordial follicle activation include the tumor suppressor phosphatase and tensin homolog (PTEN) and the transcription factor Forkhead box O3 (FOXO3) [17, 18]. Follicle development from the primordial follicle through the primary and secondary preantral follicle stages to growth and ovulation of an antral follicle is estimated to take 150–200 days in cattle [19]. Therefore, one paradigm is that events around the time of parturition and particularly bacterial infections after parturition might impact preantral follicle development, which would reduce the likelihood of conception some 8–16 weeks later, when animals are usually inseminated. The present study tested

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the hypothesis that LPS perturbs the development of primordial ovarian follicles.

MATERIALS AND METHODS

Reagents

All reagents were purchased from Sigma-Aldrich (Gillingham, U.K.) unless otherwise stated.

Ovarian Cortex Collection and Culture

Ovaries were collected from cows within 15 min of slaughter at a local slaughterhouse and transported directly to the laboratory in Medium 199 (Invitrogen, Paisley, U.K.) containing 25 mM HEPES, 2.5 μg/ml pyruvic acid, and 1% penicillin-streptomycin at 33°C–37°C. Ovaries were rinsed in 70% ethanol, followed by a brief rinse in sterile phosphate-buffered saline (PBS) and maintained at 37°C until cortical tissue cultures were prepared, as described previously [20]. Briefly, ovaries were dissected in dissection medium, consisting of Leibovitz medium containing 0.5% bovine serum albumin (BSA) fraction V, 2 mM sodium pyruvate, 3 mM L-glutamine, and 1% penicillin-streptomycin. Fine cortical tissue pieces were dissected using stereomicroscopy to generate ex vivo explants 5 mm × 5 mm wide and 1 mm thick. A maximum of two cortical pieces were collected from each ovary to reduce bias; cortical tissue was examined to confirm the absence of hemorrhage, luteal tissue, and antral follicles, and a total of 111 cortical strips were used to initiate culture.

For the evaluation of preantral follicle development, cortical tissue was immediately transferred to culture medium, consisting of McCoy 5a medium with bicarbonate supplemented with 20 mM HEPES, 10% fetal bovine serum (Biosera, Ringmer, U.K.), 3 mM L-glutamine, 1% penicillin-streptomycin, 2.5 μg/ml transferrin, 4 ng/ml selenium, 100 nM androstenedione, 10 ng/ml insulin, 50 μg/ml ascorbic acid, sodium salt. Cultures were maintained in 1 ml of culture medium in 24-well plates (TPP, Trasadingen, Switzerland) for 6 days at 37°C in humidified air with 5% CO_2. To evaluate the impact of LPS on primordial follicle development, cortical tissues were treated with control medium or media containing 0.1, 1, or 10 μg/ml ultrapure LPS from *E. coli* serotype O111:B4 (Invivogen, San Diego, CA) at the initiation of culture. Half the culture medium was collected and replaced every 48 h, including the LPS treatment.

Isolation and Culture of Individual Preantral Follicles

To examine the impact of LPS on preantral follicles beyond the primordial stage, cortical strips from bovine ovaries were prepared in dissection medium, as described above. Then, follicles with no identifiable antral cavity were identified using stereomicroscopy and sterile 26-G needles employed to manually isolate the secondary follicles from the surrounding stromal tissue. A maximum of 5 individual follicles were isolated from each ovary to reduce bias, and a total of 96 individual follicles initiated culture. Follicles were washed twice in fresh dissection medium before culture individually in 200 μl of culture medium (described above) in 96-well V-bottom plates (TPP) for 6 days at 37°C in humidified air with 5% CO_2. The preantral follicles were treated with 0, 0.1, 1, or 10 μg/ml ultrapure LPS (Invivogen) at the initiation of culture. Half the medium was collected and replaced every 48 h, including the LPS treatment.

Enzyme-Linked Immunosorbent Assay

Concentrations of IL-1β, IL-6 (Thermo Scientific, Erembodegem, Germany), IL-8 (R&D, Abingdon, U.K.), and estradiol (DRG Instruments, GmbH, Germany) were measured in cell-free supernatants, using commercially available enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions. Due to the large accumulation of IL-6 during culture, supernatants were diluted 1:25 in reagent diluent to allow accurate quantification. While the IL-1β and IL-6 ELISAs are bovine-specific, the IL-8 ELISA is cross-reactive for bovine IL-8 [21]. The limits of detection for IL-1b, IL-6, IL-8, and estradiol were 20.1, 35.6, 14.3 and 10.7 pg/ml, respectively; the intra-assay coefficients of variance were 4.6%, 1.2%, 1.7%, and 1.1%, and the interassay coefficients of variance were 7.7%, 3.0%, 5.5%, and 1.2%, respectively.

Acute LPS Exposure in Mice

To examine whether LPS impacted ovarian follicle development in vivo, wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratories (Margate, Kent, U.K.) as they provided a more tractable model than cattle. In addition, *Tlr4*$^{-/-}$ mice on the same C57BL/6 genetic background were kindly donated by Dr. C. Bryant (University of Cambridge; originally provided by S. Akira, Osaka University, Japan); these mice are insensitive to LPS and have no reported phenotype affecting fertility or ovarian development [10]. Breeding colonies were maintained under standardized conditions, in a pathogen-free environment, with access to water and standard rodent diet. All mouse procedures were conducted at the Royal Veterinary College Biological Services Unit under the U.K. Animal Scientific Procedures Act (1986), with the approval of the U.K. Government Home Office (license no. PPL 70/6424) and the Royal Veterinary College Local Ethical Review Committee.

To test the impact of LPS on ovarian follicle development in vivo, ovaries were collected 24 h after 6- to 8-week-old female WT mice were infused intraperitoneally with 100 μg of ultrapure LPS from *E. coli* serotype O111:B4 (Invivogen) or PBS (total volume of 100 μl; n = 4 to 7 per treatment), 24 h following administration of 5 IU of equine chorionic gonadotropin (Intravet, Milton Keynes, U.K.). To confirm that the effect of LPS on follicle development was mediated by the specific LPS receptor, ovaries were also collected 24 h after 6- to 8-week-old female *Tlr4*$^{-/-}$ C57BL/6 mice (n = 6) were infused intraperitoneally with 100 μg LPS in a total volume of 100 μl, 24 h after administration of 5 IU of equine chorionic gonadotropin.

Tissue Fixation, Histology, Immunofluorescence, and Microscopy

Cultured cortical strips and mouse ovaries were fixed in 10% neutral buffered formalin for 24 h. Following fixation, the tissues were dehydrated in 70% ethanol and embedded in paraffin wax. Then, 7-μm serial sections of the entire tissue were cut and mounted on glass slides. Every fourth mouse serial section and every fifth cow serial section was stained with hematoxylin and eosin, using an Auto Stainer XL (Leica, Allendale, NJ). Histological sections were analyzed on an Olympus light microscope (Olympus, Southend-on-Sea, U.K.), and each follicle was classified according to the method of Myers et al. [22] for mouse ovary and Wandji et al. [23] for bovine ovary.

A subset of bovine tissue sections underwent immunohistochemistry to localize FOXO3 and PTEN protein. Briefly, sections were dewaxed and rehydrated before antigen retrieval was performed by boiling sections in citrate buffer as described previously [24]. Rabbit anti-PTEN (catalog no. PA1-808; Thermo Scientific) and rabbit anti-FOXO (catalog no. LS-B1814; Source Bioscience, Nottingham, U.K.) were diluted 1:100 and incubated with tissue sections overnight at 4°C; as controls, some sections were processed with no primary antibody. Slides were subsequently washed, and goat anti-rabbit-Alexa 488 (Invitrogen) was diluted 1:800 and applied to tissue sections for 90 min at room temperature in the dark. Sections were mounted and coverslipped using 50% glycerol in PBS with 1 μg/ml Hoechst 33342 (Invitrogen). A minimum of two nonserial sections were stained from four cortical strips in each group.

To determine follicle and oocyte diameters, individual preantral follicles were stained in phalloidin-Alexa 555 (Invitrogen) and analyzed using confocal microscopy as described previously [25]. Briefly, individual follicles were fixed in 4% paraformaldehyde and stored in immunohistochemistry wash buffer (0.2% sodium azide, 2% normal goat serum, 1% BSA, 0.1% glycine, and 0.1% Triton X-100 in PBS) until whole-mount processing. Whole mounts were prepared by incubating each follicle in phalloidin-Alexa 555 and 1 μg/ml Hoechst 33342 in wash buffer, as described previously [26]. Follicle and oocyte diameters were measured in 6–9 individual follicles from each treatment group.

Whole-mount follicles and immunohistochemistry samples were analyzed with a model LSM 710 confocal microscope (Zeiss, Jena, Germany) using a 40× plan-apochromat objective (na = 1.3), equipped with KrArg (405 nm and 488 nm, respectively) and HeNe (543 nm) lasers, using Zen software (Zeiss) to collect fluorescence data for the blue, green, and red channels, respectively, and to generate representative images. In addition, Zen software was used to quantify PTEN and FOXO3 fluorescence intensity (arbitrary units [AU]), and to generate line scan plots to examine the cellular localization of FOXO3 and DNA. Whole-mount follicle diameter was determined by averaging two perpendicular measurements through the widest focal plane. Oocyte diameter was determined by averaging two perpendicular measurements through the center of the germinal vesicle.

Statistical Analysis

SPSS version 13.0 software was used for statistical analysis. The proportions of follicles classified as primordial, primary, secondary, antral, or atretic were compared between treatments by using the chi-square test. Concentrations of inflammatory mediators and measurements of follicle or oocyte diameter were analyzed using ANOVA, and comparisons were made with control using the Dunnett pair-wise post hoc test. Data are arithmetic

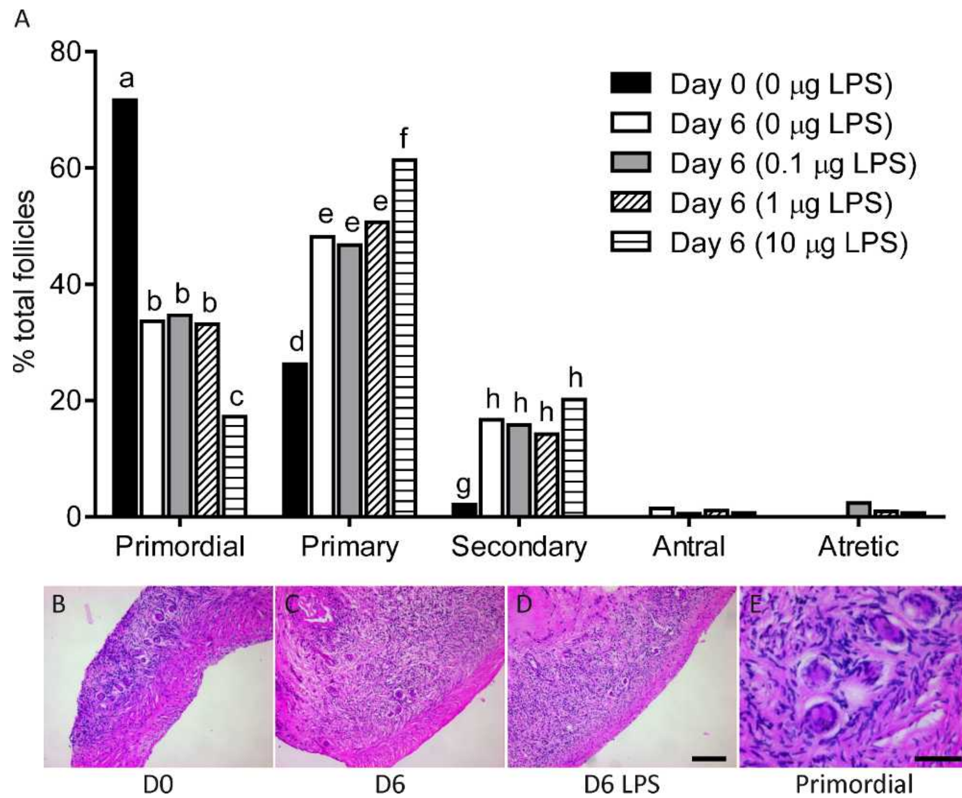

FIG. 1. Lipopolysaccharide increases primordial follicle activation in cultured bovine ovarian cortex. Bovine cortical ovarian tissue was cultured for 6 days in control medium (0) or medium containing 0.1, 1, or 10 μg/ml LPS. Total follicle counts were performed on serial sections of the entire ovarian cortical piece and compared with uncultured cortical tissue from the day of tissue collection (**A**). Representative images show uncultured cortical ovarian tissue (**B**), cortical tissue cultured for 6 days in control medium (**C**) or medium containing 10 μg/ml of LPS (**D**) and representative primordial follicles from uncultured ovarian tissue (**E**). Data are presented as the percent total follicles from each pooled group. Day 0, n = 18; Day 6 control, n = 7; Day 6 0.1 μg/ml LPS, n = 4; Day 6 1 μg/ml LPS, n = 4; Day 6 10 μg/ml LPS, n = 4. Different lowercase letters represent statistical significance following a chi square test; $P < 0.05$. Bar = 200 μm (**B**–**D**) and 50 μm (**E**).

means ± SEM, and a P value of < 0.05 was assumed to be statistically significant.

RESULTS

LPS Reduces the Primordial Follicle Pool in Bovine Ovarian Cortex Ex Vivo

To determine the effect of LPS on the primordial follicle pool, bovine ovarian cortex was cultured in control medium or media containing a range of concentrations of LPS, and preantral follicle development was evaluated and compared among treatment group and with freshly isolated, uncultured ovarian cortex (Fig. 1). Ovarian cortex was exposed to LPS at concentrations of 0.1, 1, and 10 μg/ml to mimic those measured in follicular fluid of cows with active uterine infection and those used to elicit maximal response of bovine granulosa cells in vitro [4, 14]. A total of 37 cortical strips were assessed for follicle development, and 3227 individual follicles were identified and categorized. In freshly isolated ovarian cortex, 76.0% ± 3.5% of the total follicle pool was primordial follicles, with 22.5% ± 3.2% primary and 1.5% ± 0.5% secondary stage preantral follicles. As expected, after 6 days of culture in control medium, spontaneous development from the primordial to the primary (47.0% ± 3.2%) and secondary (15.9% ± 2.5%) stages of development was evident [25]. Ovarian cortex cultured for 6 days in the presence of 0.1 and 1 μg/ml LPS showed no significant difference in the progression of follicle development compared with control cultures. However, after 6 days of culture in the presence of 10 μg/ml of LPS, a significantly higher rate of follicle activation was seen than in control cultures: only 24.6% ± 9.3% of follicles remained in the primordial stage, and there were more primary (56.7% ± 6.8%) and secondary (17.6% ± 3.2%) follicles compared than in control cultures ($P < 0.05$) (Fig. 1A).

Bovine Ovarian Cortex Produces an Inflammatory Response to LPS

Cortical ovarian tissue was cultured in the presence or absence of LPS for 6 days, with half the culture medium, including LPS, being replaced every 48 h. Cell-free supernatants were assayed after 2, 4, and 6 days of culture for the accumulation of the inflammatory cytokines IL-1β (Fig. 2, A–C) and IL-6 (Fig. 2, D–F), and the chemokine IL-8 (Fig. 2, G–I). After 2 days of culture in LPS, the supernatants had accumulated significantly more IL-1β (Fig. 2A), IL-6 (Fig. 2D), and IL-8 (Fig. 2G) than cultures in control medium ($P < 0.05$). The accumulation of IL-1β and IL-6 in culture supernatants did not differ significantly from the concentrations in control cultures following 4 or 6 days of culture (Fig. 2, B and C, and E and F, respectively). However, the LPS-stimulated accumulation of IL-8 persisted above that of control cultures even after 4 and 6 days of culture ($P < 0.05$) (Fig. 2, H and I, respectively). Concentrations of IL-6 that accumulated in cortical ovarian cultures was notably high, even in the absence of LPS stimulation (Fig. 2, D–F).

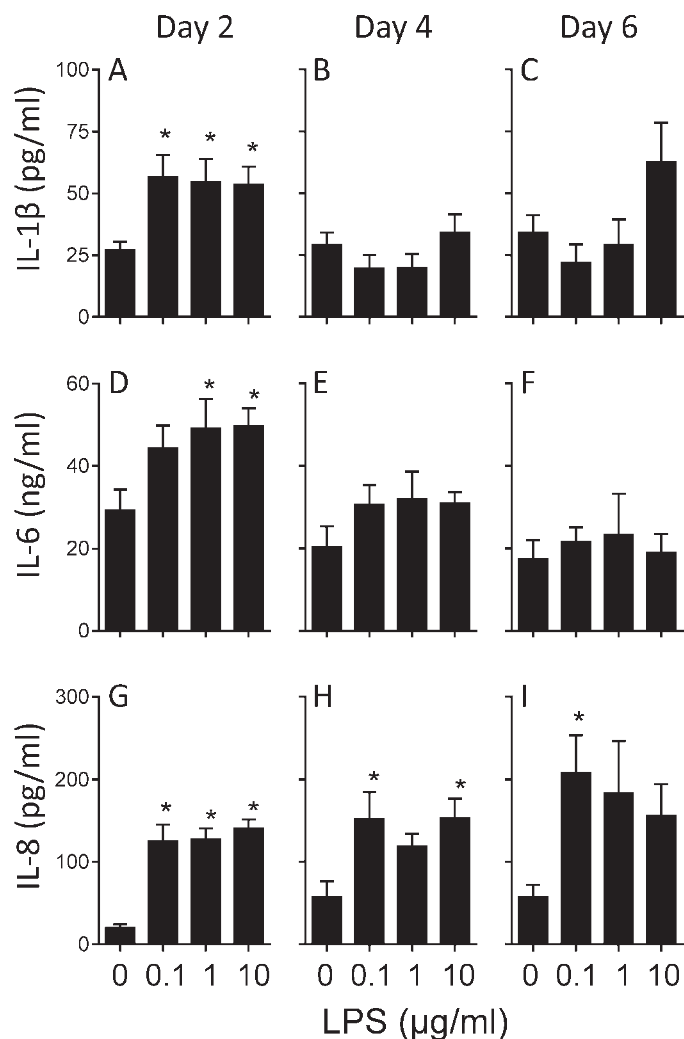

FIG. 2. Lipopolysaccharide increases accumulation of inflammatory mediators in cultured ovarian cortex. Accumulation of IL-1β (**A**–**C**), IL-6 (**D**–**F**), and IL-8 (**G**–**I**) was measured in the supernatant of bovine cortical ovarian tissue cultured for 2 (**A**, **D**, **G**), 4 (**B**, **E**, **H**), or 6 (**C**, **F**, **I**) days in control medium (0) or medium containing 0.1, 1, or 10 μg/ml LPS. Data are presented as mean + SEM from 4 independent experiments. *$P < 0.05$ compared with untreated controls; analysis by ANOVA followed by Dunnett pair-wise posthoc tests.

LPS Alters Expression of PTEN and FOXO3 Modulators of Primordial Follicle Activation in Bovine Ovarian Cortex

After 6 days of culture in control medium or media containing LPS, bovine cortical strip cultures were subjected to immunohistochemical analysis to localize the key modulators of primordial follicle activation PTEN (Fig. 3) and FOXO3 (Fig. 4).

In freshly isolated cortical tissue, PTEN was localized to primordial and primary follicle oocytes (Fig. 3, A–C). Following 6 days of culture in the absence of LPS, PTEN remained in oocytes of primordial and primary follicles (Fig. 3, D–F). However, following 6 days of culture in the presence of LPS, the remaining primordial follicles showed reduced immunoreactivity for PTEN (Fig. 3, G–I). Assessment of fluorescence intensity values further validated the loss of PTEN immunoreactivity in cultures exposed to the highest dose of LPS (uncultured, 30.7 ± 2.1 AU; Day 6 control, 46.1 ± 3.3 AU; Day 6 LPS, 10 μg/ml, 18.6 ± 1.1 AU; $P < 0.05$; also see Supplemental Fig. S1, available online at www.biolreprod.org).

In freshly isolated cortical tissue, FOXO3 was localized to the nuclei of primordial and primary follicles (Fig. 4, A–C). After 6 days of culture in control medium, FOXO3 remained localized to the nuclei of oocytes in primordial and primary follicles (Fig. 4, D–F; Supplemental Fig. S2, line scan plots). However, after 6 days of culture in the presence of LPS, oocytes in primordial follicles showed either a complete loss of FOXO3 immunoreactivity or translocation of FOXO3 out of the nucleus (Fig. 4, G–I; Supplemental Figs. S1 and S2). Assessment of fluorescence intensity values further validated the loss of FOXO3 immunoreactivity in cultures exposed to the highest dose of LPS (uncultured, 67.7 ± 6.5 AU; Day 6 control, 50.7 ± 4.6 AU; Day 6 LPS 10 μg/ml, 33.6 ± 5.6 AU; $P < 0.05$) (Supplemental Fig. S1).

LPS Does Not Impact Cultured Preantral Follicles Beyond the Primordial Stage

Estradiol production is reduced and IL-6 accumulation increased in response to LPS treatment of granulosa cells isolated from antral follicles larger than 4 mm in diameter [4, 14]. To examine the impact of LPS on preantral, secondary follicles, the production of estradiol and IL-6 by individual cultured secondary follicles was assessed in control medium or medium containing a range of concentrations of LPS (Fig. 5). Cultured preantral follicles in the absence of LPS produced 96.7 ± 20.5 pg/ml of estradiol after 2 days of culture and maintained estradiol production after 6 days of culture, consistent with continued viability. The presence of LPS had no significant effect on estradiol production by preantral follicles after 2 days (Fig. 5A) or 6 days of culture (Fig. 5B).

The concentration of IL-6 in the supernatants of cultured preantral follicles in the absence of LPS was 162.7 ± 26.1 pg/ml after 2 days and 89.2 ± 7.8 pg/ml after 6 days. There was no significant effect of LPS on the production of IL-6 after 2 days (Fig. 5C), but the addition of 1 μg/ml LPS increased IL-6 production by preantral follicles to 208.6 ± 42.5 pg/ml after 6 days of culture (Fig. 5D).

Following 6 days of culture, preantral follicles were prepared as whole mounts to assess follicle and oocyte diameter by confocal microscopy. Following 6 days of culture, mean follicle diameter was 388.7 ± 36.4 μm, and this was unchanged in the presence of LPS (Fig. 6A). Similarly, the mean oocyte diameter of follicles cultured in the absence of LPS was 73.2 ± 12.0 μm, and this was not significantly changed by LPS (Fig. 6B). A surrogate marker of oocyte competence is the synchronization of oocyte and follicle growth, evaluated by calculating the follicle/oocyte diameter ratio; although this value was not significantly different among the treatment groups (Fig. 6C).

LPS Reduces the Primordial Follicle Pool in Murine Ovaries In Vivo

To examine whether LPS impacted total ovarian follicle reserve in vivo, ovaries of WT C57BL/6 mice infused with LPS were compared with those infused with PBS. As expected, control WT mice receiving PBS (Fig. 7, A and B) had predominantly primordial (65.4 ± 2.6%) and primary (12.2% ± 1.8%) ovarian follicles, with little follicle atresia (3.0% ± 0.5%). However, when WT mice received a single infusion of LPS (Fig. 7, A and C), follicle atresia 3.3-fold increased compared with that of control animals (9.8% ± 1.0%, $P < 0.05$). As a result of the increased follicle atresia, the primordial follicle pool was 1.2-fold reduced compared that of control animals (56.4% ± 1.8%, $P < 0.05$).

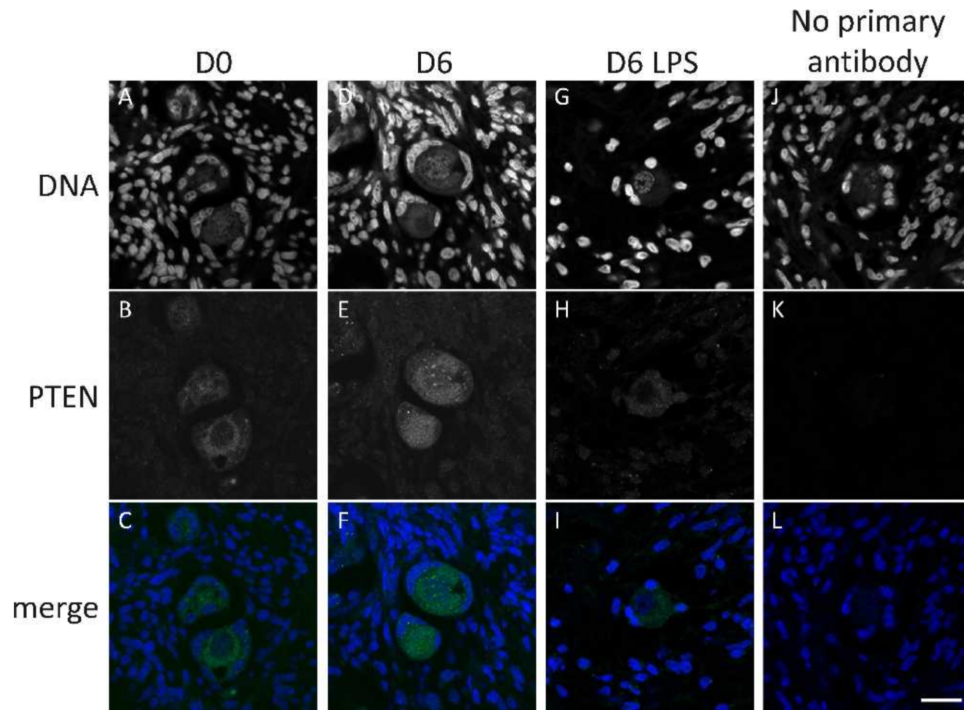

FIG. 3. Lipopolysaccharide reduces primordial follicle PTEN in cultured ovarian cortex. Bovine cortical ovarian tissue was cultured for 6 days in the absence (**D**–**F**) or presence of 10 μg/ml LPS (**G**–**I**) and compared with uncultured cortical tissue from the day of tissue collection (**A**–**C**). Immunolocalization of PTEN was performed on fixed tissues and assessed by confocal microscopy. Representative confocal images include primordial and transition follicles showing DNA (blue), PTEN (green), and a merge of the two color channels. Representative images of sections where no primary antibody was applied are shown in **J**, **K**, and **L**. Bar = 25 μm.

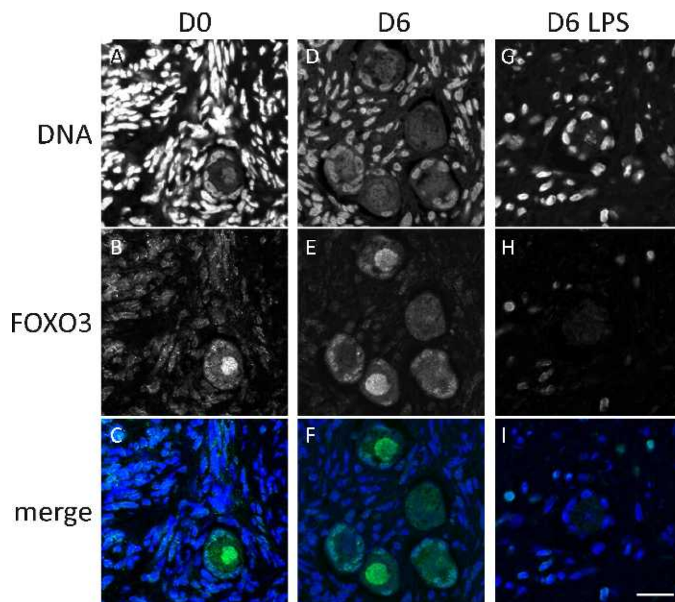

FIG. 4. Lipopolysaccharide alters primordial follicle FOXO3 expression and localization in cultured ovarian cortex. Bovine cortical ovarian tissue was cultured for 6 days in the absence (**D**–**F**) or presence of 10 μg/ml LPS (**G**–**I**) and compared with uncultured cortical tissue from the day of tissue collection (**A**–**C**). Immunolocalization of FOXO3 was performed on fixed tissues and assessed by confocal microscopy. Representative confocal images include primordial and transition follicles showing DNA (blue), FOXO3 (green), and a merge of the two color channels. Bar = 25 μm.

Ovaries were also collected from *Tlr4*$^{-/-}$ C57BL/6 mice infused with LPS to confirm that the impact of LPS on follicle development was mediated via TLR4, which is the specific receptor for LPS. As expected, there was no significant difference in the follicle reserve of *Tlr4*$^{-/-}$ mice receiving LPS compared with WT mice receiving vehicle only (Fig. 7, A and D).

DISCUSSION

Primordial follicles containing an oocyte surrounded by a single layer of pregranulosa cells are formed during fetal life in ruminants [19]. Many primordial follicles wait several years before undefined factors signal a resumption of development for a cohort of follicles through gonadotropin-independent preantral stages toward antral follicles; some of which are recruited into follicular waves, leading to selection of a dominant follicle and, potentially, ovulation to form a zygote. A fundamental question in reproductive biology is whether oocytes are impacted by external factors such as microbial infections. While infection of the ovary itself is a rare event, bacterial infections of the reproductive tract or mammary gland are common following parturition in dairy cattle. These infections perturb ovarian antral follicle growth and function [3, 5, 6]. During uterine disease, LPS is evident within follicular fluid in vivo, and LPS elicits an inflammatory response from granulosa cells in antral follicles or cumulus-oocyte complexes in vitro, which also perturbs their endocrine function [4, 14]. However, even after the resolution of bacterial infections, animals still have lower conception rates [1, 2, 7]. Here, we proposed that exposure to LPS during uterine infection has longer term consequences on the follicle reserve, leading to infertility following resolution of infection. In the

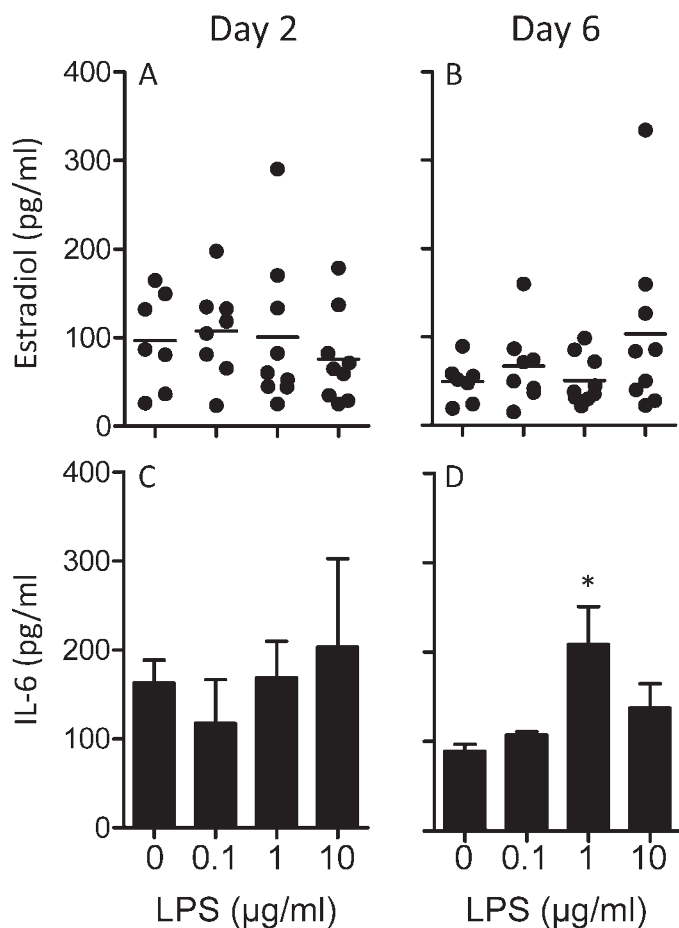

FIG. 5. Lipopolysaccharide does not affect estradiol or IL-6 accumulation in cultured preantral follicles. Accumulation of estradiol (**A**–**B**) and IL-6 (**C**–**D**) was measured in the supernatant of individual bovine preantral follicles cultured for 2 (**A**, **C**) or 6 (**B**, **D**) days in control medium (0) or medium containing 0.1, 1, or 10 µg/ml LPS. Data are presented as mean + SEM. *$P < 0.05$ compared with untreated controls; analysis by ANOVA followed by Dunnett pair-wise posthoc tests.

present study, LPS reduced the primordial follicle reserve in cattle and mice, with evidence of inflammation and dependence on the TLR4 receptor for LPS.

Cortical ovarian tissue culture was used to determine the effect of LPS on ovarian follicle reserve in cattle. As expected, the culture of ovarian cortex activated a proportion of the primordial follicles in control medium [20]. However, primordial follicle activation was further accelerated in ovarian cortical tissue cultured in the presence of the highest concentration of LPS, with increased proportions of primary and secondary follicles. These findings imply that inappropriate premature activation of primordial follicles would likely deplete ovarian follicle reserve and, thus, compromise subsequent fertility. Although, the LPS concentrations were similar to those given in other in vitro studies using tissue explants and likely are required to penetrate into the cortical tissue [27]; the concentrations of LPS required in vitro to cause this effect are relatively high compared with the concentrations of LPS found in follicular fluid in vivo [4]. Indeed, it would be catastrophic if primordial follicles were sensitive to low concentrations of pathogen-associated molecules because multiple minor infections would rapidly deplete the follicle reserve. The impact of LPS on follicle activation in the present study agrees with well-established observations in cattle that clinical problems of the postpartum uterus are associated with compromised reproductive performance ~8–16 weeks later [28, 29]. The longer-term association between bacterial infections and premature ovarian failure are rarely tested because cattle are usually culled before reaching old age. However, the number of healthy primordial follicles declines to near zero in 20-year-old cattle from ~140 000 in 4- to 6-year-old animals [30]; and, cattle with long-term mastitis had more emerged 5- to 9-mm-diameter follicles at estrus compared to those of uninfected cows [31]. Although, chronic mastitis had no effect on the primordial or primary follicle pool as assessed by examining a small, 56-mm^2 area of the ovary and without comparison to uninfected animals [32]. Further investigations are warranted as depletion of the primordial follicle pool would impact the ovarian reserve of older animals and/or the superovulation response of oocyte donor animals.

Initial activation of primordial follicles requires accumulation of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) to

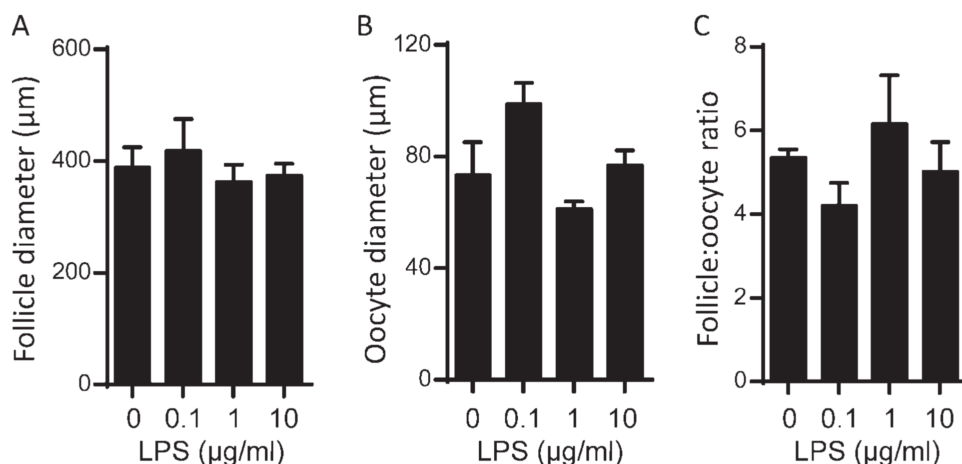

FIG. 6. Lipopolysaccharide does not affect follicle or oocyte growth in cultured preantral follicles. Follicle (**A**) and oocyte (**B**) diameter was measured in individual bovine preantral follicles cultured for 6 days in control medium (0) or medium containing 0.1, 1, or 10 µg/ml LPS. Following phalloidin staining and whole mounting follicles were imaged by confocal microscopy. Images were collected at the largest cross sectional area of the follicle and in the middle of the germinal vesicle of the oocyte. Two perpendicular measurements were made from the basement membrane in follicles or the outer zona in oocytes and averaged to estimate diameter. Follicle: oocyte diameter ratio (**C**) was used as a surrogate measure of synchronized oocyte/ follicle growth. Data are presented as mean + SEM. *$P < 0.05$ compared with untreated controls; analysis by ANOVA followed by Dunnett pair-wise posthoc tests.

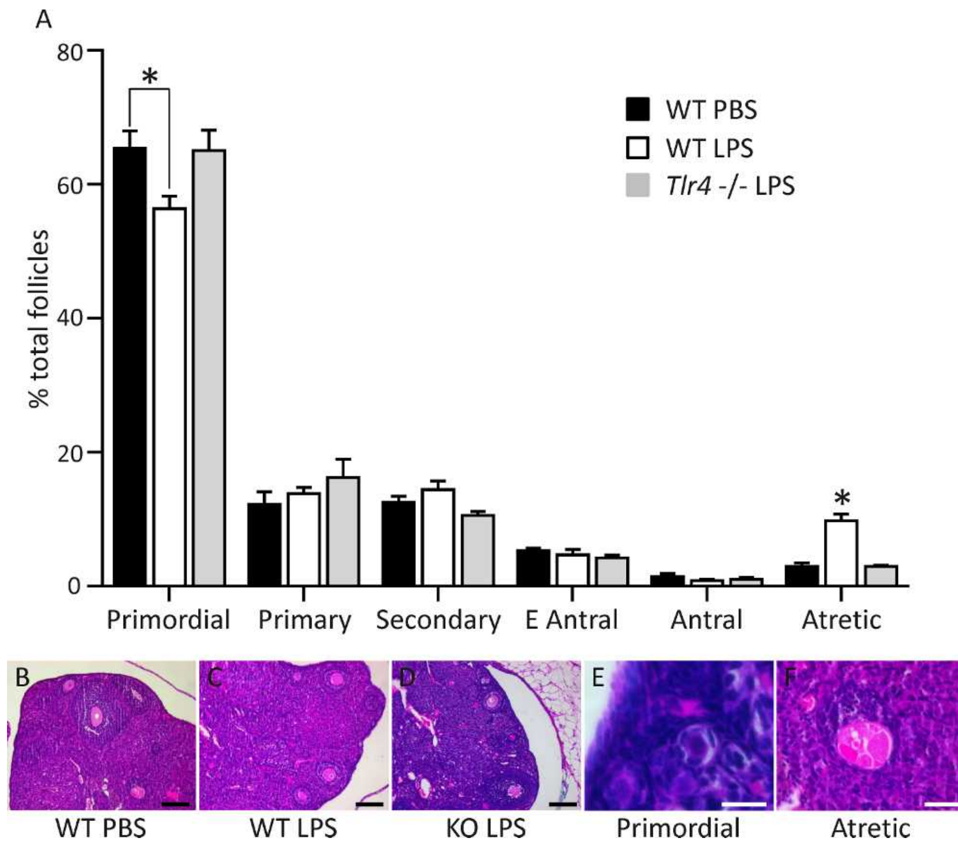

FIG. 7. Acute lipopolysaccharide exposure of mice increases follicle atresia in a TLR4 dependent manner. Wild-type (WT) or *Tlr4*$^{-/-}$ mice received either vehicle (PBS) or LPS 24 h prior to collection of tissues. Total follicle counts were performed on serial sections of the entire ovary (**A**). Representative images show ovarian morphology of vehicle treated wild-type (**B**), LPS treated wild-type (**C**) or LPS treated *Tlr4*$^{-/-}$ (**D**) mice. Representative images of primordial (**E**) and atretic (**F**) follicles are shown. Data are presented as the mean + SEM percent total follicles from each animal. *$P < 0.05$ compared with other groups within a follicle classification; analysis by a chi square test. Bar = 200 μm (**B–D**), 25 μm (**E**), and 50 μm (**F**).

allow the progression of the downstream Akt/phosphokinase-B (AKT/PKB) pathway [33]. Both PTEN and FOXO3 are involved in regulating the AKT/PKB pathway, integral to primordial follicle activation in the mouse [18, 34]. Studies in knockout mice have confirmed that PTEN and FOXO3 are negative regulators of follicle activation [17, 35]. In the mouse, PTEN maintains reduced levels of PIP_3 in the follicle and restrains the follicle in a quiescent state, effectively acting as a negative regulator of follicle activation. When PTEN levels fall, PIP_3 accumulates and primordial follicles are activated. Similarly, in the present study, follicle activation in bovine ovarian cortex was associated with reduced expression of the PTEN protein following 6 days' treatment with LPS. While PTEN has not been studied in the bovine ovary, FOXO3 knockdown in bovine ovarian cultures has been shown to promote the activation of primordial follicles [36]. In the present study, the primordial follicle activation following treatment of ovarian cortex with LPS was also associated with translocation of FOXO3 out of the nucleus. Of course, while primordial follicle loss of PTEN and cytoplasmic translocation of FOXO3 were increased following exposure to LPS in the present study, an alternative consideration is that these changes may reflect increased primordial activation as opposed to a mechanism of LPS action leading to increased primordial activation. In this light, IL-6 has been shown to independently induce the AKT/PKB pathway in immune cells, while reduction of PTEN increases the IL-6 feed forward loop in cultured cell lines [37, 38]. In cortical tissues cultured in the absence of LPS, IL-6 concentrations were notably high (>20 ng/ml). These high concentrations may be enough to override the negative regulation of the Akt pathway by PTEN and may explain, in part, the spontaneous activation of primordial follicles in cultured ovarian cortex. Attempts to block IL-6 signaling in cortical cultures using the STAT3 inhibitor Static failed to reduce IL-6 accumulation and was not pursued (data not shown). Support for a direct action of infection on follicle development comes from a recent study of the growth differentiation factor 9, which is critical for early folliculogenesis and was less abundant within the oocytes of antral follicles in animals with a severe form of chronic mastitis [32].

An obvious feature to explore while evaluating primordial follicle activation was the production of the inflammatory mediators typically associated with the innate immune response to LPS [8, 9]. Indeed, the cytokines IL-1β and IL-6 and the chemokine IL-8 accumulated in the cortical strip culture medium after 2 days' treatment with LPS, with the IL-8 response persisting for up to 6 days of culture. Concentrations of IL-6 in the supernatants of bovine ovarian cortical strips treated with LPS were markedly higher than in the supernatants following LPS treatment of granulosa cells isolated from antral follicles or LPS treatment of in vitro organ cultures of bovine endometrium [14, 27]. It would be interesting to explore how IL-6 production by the ovary is constrained in vivo and why this is not the case ex vivo.

Following LPS-induced primordial follicle activation, the next obvious question was whether LPS might impact the subsequent steps in preantral follicle development. Once follicles have left the pool of primordial follicles, they are

committed to gonadotropin-independent growth of the oocyte surrounded by layers of granulosa cells. Granulosa cells collected from dominant and growing antral follicles mount an inflammatory response to LPS, including accumulation of IL-6 and perturbation of estradiol production [4, 14]. However, when preantral follicles were cultured individually in the present study, LPS did not significantly affect either IL-6 or estradiol production or the growth parameters of the follicle and enclosed oocyte. There are several potential explanations for this difference between antral and preantral follicles. There may be a point in follicle development when granulosa cells are protected from LPS either by passive mechanisms such as formation of the basement membrane of follicles or active mechanisms that might regulate TLR4 pathway function in follicular cells. Conversely, in vitro culture of individual preantral follicles may not optimally model the impact of LPS compared with ex vivo ovarian cortex or when the ovary is exposed to LPS in vivo.

To further explore the effect of LPS on the ovarian primordial follicle pool, C57BL/6 WT mice were infused with LPS or PBS in vivo. Similar to cultured bovine tissue, WT mice given a single injection of LPS had a reduced primordial follicle pool compared with those infused with PBS, but the loss of primordial follicles was accompanied by increased follicle atresia. Interestingly, exposure of rats to LPS for 3 days also leads to an increased rate of follicle atresia [39]. In comparison to the bovine model, where only presecondary follicles are placed into culture, the entire ovarian reserve is exposed to LPS in the mouse; this may explain in part why follicle atresia was more evident in the mouse model, where larger follicles could undergo follicle atresia and be assessed before regression. To confirm that the impact of LPS on follicle development in WT mice was mediated by the specific LPS receptor, we took advantage of *Tlr4*$^{-/-}$ mice on the same C57BL/6 genetic background, which are insensitive to LPS and have no reported phenotype affecting fertility or ovarian development [10]. Indeed, infusion of LPS, as expected, did not cause any detectable treatment effect in these *Tlr4*$^{-/-}$ mice.

Previous studies have identified acute effects of infection on gonadotropin-dependent large antral follicles and ovarian endocrine function in the cow [3, 4, 6, 14]. Disruption of the hypothalamus-pituitary-ovary axis in cows with infection has also been reported and may suggest longer-term dysfunction of the ovary following resolution of infection [40]. The present study has started to uncover mechanisms underlying the impact of bacterial infection on early gonadotropin-independent follicular development in the cow. Because of the multifactorial perturbations of metritis and mastitis on the ovary, endometrium, hypothalamus-pituitary axis, and immune system, all of these systems in concert most likely result in the observed infertility of cows following resolution of infection. However, of all the possibilities, loss of the primordial follicle pool is irreversible and likely has the longest duration of effect and might even contribute to premature ovarian failure. The present study has wider relevance than just dairy cattle because bacterial infections of the uterus in women also cause long-term as well as short-term infertility [1, 41]. Furthermore, the present study may open a new avenue for investigation into whether microbial infections contribute to depletion of follicle reserve in women leading to premature ovarian failure.

In conclusion, LPS reduced the primordial follicle pool in bovine ovarian cortex ex vivo and in mice in vivo. The loss of primordial follicles was associated with increased primordial follicle activation and an inflamed ovarian environment in bovine tissues treated with LPS. In, mice LPS increased follicle atresia, and this was TLR4-dependent. These findings give insights into mechanisms of accelerated primordial follicle depletion and infertility after the resolution of bacterial infections at sites distant from the ovary.

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