Cholesterol supports bovine granulosa cell inflammatory responses to lipopolysaccharide

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Abstract

During bacterial infections of the bovine uterus or mammary gland, ovarian granulosa cells mount inflammatory responses to lipopolysaccharide (LPS). *In vitro*, LPS stimulates granulosa cell secretion of the cytokines IL-1α and IL-1β, and the chemokine IL-8. These LPS-stimulated inflammatory responses depend on culturing granulosa cells with serum, but the mechanism is unclear. Here we tested the hypothesis that cholesterol supports inflammatory responses to LPS in bovine granulosa cells. We used granulosa cells isolated from 4–8 mm and > 8.5 mm diameter ovarian follicles and manipulated the availability of cholesterol. We found that serum or follicular fluid containing cholesterol increased LPS-stimulated secretion of IL-1α and IL-1β from granulosa cells. Conversely, depleting cholesterol using methyl-β-cyclodextrin diminished LPS-stimulated secretion of IL-1α, IL-1β and IL-8 from granulosa cells cultured in serum. Follicular fluid contained more high-density lipoprotein cholesterol than low-density lipoprotein cholesterol, and granulosa cells expressed the receptor for high-density lipoprotein, scavenger receptor class B member 1 (SCARB1). Furthermore, culturing granulosa cells with high-density lipoprotein cholesterol, but not low-density lipoprotein or very low-density lipoprotein cholesterol, increased LPS-stimulated inflammation in granulosa cells. Cholesterol biosynthesis also played a role in granulosa cell inflammation because RNA interference of mevalonate pathway enzymes inhibited LPS-stimulated inflammation. Finally, treatment with follicle-stimulating hormone, but not luteinizing hormone, increased LPS-stimulated granulosa cell inflammation, and follicle-stimulating hormone increased SCARB1 protein. However, changes in inflammation were not associated with changes in oestradiol or progesterone secretion. Taken together these findings imply that cholesterol supports inflammatory responses to LPS in granulosa cells.

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In brief: Bovine granulosa cells need to be cultured with serum to generate inflammation in response to bacterial lipopolysaccharide. This study shows that it is cholesterol that facilitates this lipopolysaccharide-stimulated cytokine secretion.

Introduction

Bacterial infections of the postpartum uterus or mammary gland perturb ovarian function and reduce fertility in dairy cattle (Ribeiro, *et al.* 2016, Roth and Wolfenson 2016, Sheldon, *et al.* 2002). Ovarian follicular fluid from animals with uterine disease contains lipopolysaccharide (LPS), which is a cell wall component of Gram-negative bacteria (Herath, *et al.* 2007). Lipopolysaccharide bound to CD14 is sensed via Toll-like receptor 4 (TLR4), leading to inflammatory responses in innate immune and tissue cells (Moresco, *et al.* 2011, Sheldon, *et al.* 2019). Although healthy ovarian follicles do not contain innate immune cells, ovarian follicle granulosa cells express TLR4 and mount inflammatory responses to LPS, including the secretion of cytokines IL-1α, IL-1β and IL-6, and the chemokine IL-8 (Bromfield and Sheldon 2011, Horlock, *et al.* 2021, Price, *et al.* 2013). These inflammatory responses to LPS depend on culturing bovine granulosa cells with fetal bovine serum and we suggested that serum facilitated inflammation by supplying soluble CD14 (Bromfield and Sheldon 2011). However, granulosa cells express CD14, and LPS stimulates increased CD14 expression, which is inconsistent with depending on serum to supply soluble CD14 (Herath, *et al.* 2007, Shimizu, *et al.* 2012). In the present study we considered whether there was an alternative explanation for serum supporting LPS-stimulated inflammation in granulosa cells.

As well as the need for cholesterol to make plasma membranes in proliferating cells, such as granulosa cells, cholesterol promotes inflammatory responses in innate immune cells (Dang, *et al.* 2017, Tall and Yvan-Charvet 2015). Plasma membrane cholesterol is thought to be important for TLR4 signalling because the cholesterol-depleting agent, methyl-β-cyclodextrin, inhibits LPS-stimulated inflammation in human monocytes (Triantafilou, *et al.* 2002). Serum cholesterol is principally bound to high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL). Lipoproteins are complex particles typically composed of 80–100 proteins that transport cholesterol. Cells use scavenger receptor class B member 1 (SCARB1, also known as SR-B1) to take up HDL cholesterol, and the LDL receptor (LDLR) for LDL cholesterol (Acton, *et al.* 1996, Brown and Goldstein 1976). Bovine granulosa cells express *SCARB1* and *LDLR* mRNA, and *SCARB1* mRNA expression increases after 72 h of culture (Yamashita, *et al.* 2011). However, ovarian follicle basement membranes are only permeable to proteins up to 300 kDa, which excludes LDL (~3,500 kDa) and VLDL (6,000 to 27,000 kDa), leaving HDL (175 to 500 kDa) as the predominant lipoprotein in follicular fluid with concentrations 30 to 45% of

serum concentrations (Brantmeier, *et al.* 1987, Savion, *et al.* 1982, Siu and Cheng 2012). Postpartum cows have reduced serum HDL, LDL and VLDL cholesterol, and changes in serum cholesterol concentrations are reflected in follicular fluid (Kessler, *et al.* 2014, Leroy, *et al.* 2004). However, bovine granulosa cells can also synthesise cholesterol via the mevalonate pathway, controlled by the rate limiting enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), and follicle-stimulating hormone (FSH) stimulates cholesterol biosynthesis (Bertevello, *et al.* 2018, Reverchon, *et al.* 2014). At least in rat granulosa cells, FSH also increases SCARB1 uptake of HDL to evade cholesterol homeostatic feedback control during steroidogenesis (Lai, *et al.* 2013). Interestingly, FSH also increases granulosa cell inflammatory responses to LPS (Bromfield and Sheldon 2011). Therefore, we considered whether granulosa cell inflammatory responses to LPS might also be facilitated by cholesterol.

Here we tested the hypothesis that cholesterol supports inflammatory responses to LPS in bovine granulosa cells. We first determined whether granulosa cell IL-1α, IL-1β and IL-8 responses to LPS were altered by supplying serum or follicular fluid containing cholesterol, or by depleting cholesterol in serum. The concentration of cholesterol in follicular fluid and the abundance of SCARB1 and LDLR protein was quantified, and the effects of HDL, LDL or VLDL on LPS-stimulated granulosa cell inflammation were measured. Short-interfering RNA (siRNA) was used to examine the effect of cholesterol biosynthesis on granulosa cell responses to LPS. We evaluated whether FSH could increase granulosa cell inflammatory responses to LPS, and the expression of SCARB1 and HMGCR protein. In addition, we explored whether changes in inflammation were associated with changes in oestradiol or progesterone secretion.

Materials and methods

Ethical statement

Ovaries were collected from cattle after slaughter and processing with approval from the United Kingdom Department for Environment, Food and Rural Affairs under the Animal By-products Registration (EC) No. 1069/2009 (registration number U1268379/ABP/OTHER).

Granulosa cell culture

Ovaries were collected within 15 min of slaughter from post pubertal, non-pregnant, healthy mixed-breed beef heifers. Ovaries were transported to the laboratory within 90 min in

Medium 199 (M199; Thermo Fisher Scientific, Paisley, UK) supplemented with 1% Antibiotic Antimycotic Solution (ABAM; Merck) and 0.1% bovine serum albumin (BSA; Merck) at 38.5°C. Granulosa cells were isolated and cultured as described previously (Horlock*, et al.* 2021, Price*, et al.* 2013). Briefly, ovaries were first rinsed in 70% ethanol and then phosphate buffered saline (PBS; Thermo Fisher Scientific). Using a sterile 20-gauge needle and 5 ml LPS-free syringe (BD Medical, Oxford, UK), mural granulosa cells were aspirated from follicles into collection medium comprising M199 supplemented with 0.5% w/v BSA, 25 mM HEPES (4-[2-hydroxyethyl] piperazine-1-ethanesulfonic acid), 1% ABAM, and 0.005% w/v heparin (all Merck). Granulosa cells were aspirated from 4–8 mm diameter ovarian follicles to represent emerged follicles, and > 8.5 mm diameter follicles to represent dominant follicles (Fortune 1994, Price*, et al.* 2013). Collecting granulosa cells by follicle aspiration resulted in pure preparations, determined by microscopy, as reported previously (Bromfield and Sheldon 2011, Nimz*, et al.* 2009).

Granulosa cells were pooled from the ovaries of 10 to 20 animals, and the cells were washed twice in granulosa cell medium (M199 culture medium; 1% ABAM; 1% Insulin-Transferrin-Selenium, Corning, Palo Alto, USA; and 2 mM glutamine, Thermo Fisher Scientific). The cells were counted and seeded into 24-well culture plates (TPP, Trasadingen, Switzerland), at a density of 750,000 cells/well in 0.5 ml/well of granulosa cell medium supplemented with 10% serum (heat-inactivated fetal bovine serum; Biosera, Ringmer, UK), as described previously (Horlock*, et al.* 2021, Price*, et al.* 2013). The concentration of oestradiol in serum was 0.04 ng/ml and the concentration of progesterone was below the limit of detection of the assay (< 0.14 ng/ml). The cells were incubated at 38.5°C, in a humidified atmosphere of air containing 5% CO_2.

Granulosa cell experiments

Granulosa cells isolated from 4–8 mm diameter or > 8.5 mm diameter ovarian follicles were cultured in 24-well plates for 18 h to achieve 80% confluence. The culture medium was discarded, and cells were then cultured for 24 h with the treatments and media described below. The cells were finally challenged for a further 24 h using control medium or medium containing 1 µg/ml LPS (ultrapure LPS from *Escherichia coli* serotype 0111: B4, InvivoGen, Toulouse, France), in the continuing presence of the treatments (Supplementary Figure 1). At the end of each experiment, supernatants were collected to measure IL-1α, IL-1β and IL-8 by ELISA, and cell viability was evaluated by MTT assay or cells were collected for protein or

cholesterol analysis. The LPS concentration and duration of challenge were based on previous studies (Bromfield and Sheldon 2011, Horlock*, et al.* 2021, Price*, et al.* 2013). Cells were not supplied with androstenedione, except where indicated, because the aim was to examine whether cholesterol supports inflammatory responses to LPS; unlike previous studies, where androstenedione was supplied to the cells to examine whether LPS altered steroidogenesis (Herath*, et al.* 2007, Price*, et al.* 2013, Shimizu*, et al.* 2012). Although treating granulosa cells with exogenous oestradiol or progesterone does not significantly alter the accumulation of IL-6 in response to LPS (Price and Sheldon 2013), we measured oestradiol and progesterone in selected experiments to explore whether changes in steroid concentrations were associated with inflammation. Each experiment was performed with 3 to 6 independent cultures of granulosa cells, using one culture well for each treatment. All assays used two technical replicates for each sample.

To evaluate the effect of serum or follicular fluid on LPS-stimulated inflammation, granulosa cells were cultured for 24 h in granulosa cell medium and treated with a range of concentrations from 0 to 10% serum, or from 0 to 5% follicular fluid that had been aspirated and pooled from > 8.5 mm diameter ovarian follicles of 10 ovaries.

To determine the effect of depleting cholesterol from the culture medium on LPS-stimulated inflammation, granulosa cells were cultured for 24 h in granulosa cell medium supplemented with 10% serum and treated with a range of concentrations from 0 to 1 mM methyl-β-cyclodextrin (Merck), which binds to cholesterol (Christian*, et al.* 1997). The concentrations of methyl-β-cyclodextrin were based on previous studies in bovine cells (Griffin*, et al.* 2017). In preliminary experiments, treating granulosa cells with 1 mM methyl-β-cyclodextrin, without an LPS challenge, did not stimulate secretion of IL-1α, IL-1β or IL-8 (values did not differ significantly from vehicle). To determine whether granulosa cells express SCARB1 or LDLR protein, granulosa cells were cultured for 24 h in granulosa cell medium supplemented with 10% serum, and the cells were collected for Western blot analysis.

To determine the effect of cholesterol on LPS-stimulated inflammation, cells were cultured for 24 h in serum-free granulosa cell medium and treated with a range of concentrations from 0 to 100 μg/ml human HDL cholesterol (Merck), from 0 to 50 μg/ml human LDL cholesterol (Thermo Fisher Scientific), or from 0 to 10 μg/ml human VLDL cholesterol (Merck). Human HDL and LDL cholesterol have been used previously to investigate steroidogenesis in bovine, human and rat granulosa cells (Azhar*, et al.* 1998, Reaven*, et al.* 1995, Savion*, et al.* 1981). In

preliminary experiments, treating granulosa cells with lipoproteins, without an LPS challenge, did not stimulate secretion of IL-1α, IL-1β or IL-8 (values did not differ significantly from vehicle). Concentrations of HDL and LDL were similar to those used with bovine ovarian cells previously (Bao, *et al.* 1997, Carroll, *et al.* 1992, Zhang, *et al.* 2015).

To investigate whether gonadotrophins altered LPS-stimulated inflammation, granulosa cells were cultured in granulosa cell medium with or without 10% serum, and treated for 24 h with vehicle, a supraphysiological concentration of 2.5 μg/ml highly purified bovine FSH (A. F. Parlow, National Hormone and Peptide program, Torrance, California), 10^{-7} M androstenedione (Merck), or 2.5 μg/ml FSH and 10^{-7} M androstenedione in combination. These concentrations of FSH and androstenedione were based on previous bovine granulosa cell studies (Bromfield and Sheldon 2011, Gong, *et al.* 1993, Gutierrez, *et al.* 1997). Alternatively, granulosa cells isolated from > 8.5 mm diameter ovarian follicles were cultured in serum-free granulosa cell medium and treated for 24 h with a range of concentrations from 0 to 1000 ng/ml FSH or from 0 to 10 ng/ml highly purified bovine luteinizing hormone (LH; A. F. Parlow). The concentrations of FSH ranged from physiological to supraphysiological concentrations (Lainé, *et al.* 2019); the concentrations of LH were based on physiological concentrations in follicular fluid (Fortune and Hansel 1985). To determine the effect of FSH on SCARB1 and HMGCR protein, granulosa cells were cultured in granulosa cell medium supplemented with 0%, 2% or 10% serum and treated for 48 h with vehicle, 2.5 μg/ml FSH, 10^{-7} M androstenedione, or 2.5 μg/ml FSH and 10^{-7} M androstenedione in combination, and the cells were collected for Western blot analysis.

Short interfering RNA

To examine the role of cholesterol biosynthesis in LPS-stimulated inflammation, we took advantage of previously described siRNA targeting the cholesterol biosynthesis enzymes *HMGCR*, *FDPS* (encoding farnesyl pyrophosphate synthase) or *FDFT1* (encoding farnesyl-diphosphate farnesyltransferase 1) (Griffin, *et al.* 2018, Griffin, *et al.* 2017, Healey, *et al.* 2016). Cells were transfected using scramble siRNA (Horizon Discovery) or siRNA targeting *HMGCR* (sense, CAGCAUGGAUAUUGAACAAUU; antisense, UUGUUCAAUAUCCAUGCUG), *FDPS* (sense, GCACAGACAUCCAGGACAAUU; antisense, UUGUCCUGGAUGUCUGUGCUU), or *FDFT1* (sense, GCGAGAAGGGAGAGAGUUUUU; antisense, AAACUCUCUCCCUUCUCGC). Briefly, 750,000 cells/well in 24-well culture plates were cultured for 30 min in 900 μl of antibiotic-

free granulosa cell medium supplemented with 10% serum, prior to the addition of 100 µl OptiMEM medium (Thermo Fisher Scientific), containing 20 pmol siRNA and 1.5 µl Lipofectamine RNA-iMax (Invitrogen) for 24 h. Following transfection, the medium was removed, and the granulosa cells challenged for 24 h in granulosa cell medium supplemented with 10% serum with or without 1 µg/ml LPS. At the end of each experiment, cell supernatants were collected to measure IL-1α, IL-1β and IL-8 by ELISA, cell viability was estimated using the MTT assay, and protein was collected for Western blot analysis.

Although the siRNA were previously validated in bovine endometrial cells (Griffin*, et al.* 2018, Griffin*, et al.* 2017, Healey*, et al.* 2016), to quantify the efficiency of the siRNA, pooled populations of granulosa cells from 4-8 mm and > 8.5 diameter ovarian follicles were transfected with each siRNA for 48 h, and the RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen, GmbH), according to the manufacturer's instructions. The quality and quantity of RNA was assessed using a NanoDrop (ND-100 Spectrometer, Labtech International, Uckfield, UK), prior to conversion to cDNA using the QuantiTect Reverse Transcription kit (Qiagen). Quantitative PCR was carried out in triplicate with the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) using a QuantiFast SYBR Green PCR kit (Qiagen) and previously described primers for the target genes *HMGCR*, *FDPS* and *FDFT1*, and the reference genes *ACTB* and *RLP19*, as described previously (Cronin*, et al.* 2016, Griffin*, et al.* 2017). The ACTB and RPL19 reference genes did not differ in expression with treatment, and were amplified at the same efficiency as the target gene. Data were analysed using the CFX Manager Software (Bio-Rad), and target gene mRNA expression normalised to the two reference genes.

ELISA

Bovine IL-1α ELISA was performed in duplicate as described previously (Healy*, et al.* 2014, Horlock*, et al.* 2021); using polyclonal rabbit anti-bovine IL-1α capture antibody (Kingfisher Biotech, Saint Paul, USA; RRID: AB_2833237), recombinant IL-1α protein (Kingfisher Biotech, #RP0097B), biotinylated polyclonal anti-bovine IL-1α (Kingfisher Biotech; RRID: AB_2833238) and avidin HRP (Fisher Scientific; #18410051). Bovine IL-1β ELISA was performed in duplicate using a kit according to the manufacturer's instructions (Thermo Fisher Scientific; RRID AB_2833244). Bovine IL-8 ELISA was performed in duplicate as described previously (Cronin*, et al.* 2015, Horlock*, et al.* 2021); using monoclonal anti-ovine IL-8 capture antibody (Bio-Rad, Hercules, CA, USA; RRID: AB_322152), recombinant IL-8

protein (Kingfisher Biotech, #RP0023B), polyclonal rabbit anti-sheep IL-8 detection antibody (Bio-Rad; RRID: AB_322153) and HRP-conjugated goat anti-rabbit antibody (Dako, Glostrup, Denmark; RRID: AB_2617138). Assays were performed using two technical replicates for each sample. Limits of detection were 12.5 pg/ml for IL-1α, 31.3 pg/ml for IL-1β, and 62.5 pg/ml for IL-8; the inter-assay and intra-assay coefficients of variation were all < 7% and < 9%, respectively.

Oestradiol ELISA was performed in duplicate using a kit according to the manufacturer's instructions (DRG Diagnostics, Marburg, Germany; RRID AB_ 2889185). The limit of detection for oestradiol was 10.6 pg/ml, and the inter- and intra-assay CV were 1.4% and 3.7%, respectively. Progesterone ELISA was performed in duplicate using a kit according to the manufacturer's instructions (DRG Diagnostics. RRID: AB_2833253). The limit of detection for progesterone was 0.14 ng/ml, and the inter- and intra-assay CV were 2.5% and 4.4%, respectively.

MTT Assay

Granulosa cell MTT assays were performed as described previously (Horlock*, et al.* 2021). Briefly, after supernatants were collected, cells were incubated for 1 h in granulosa cell medium containing 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)12,5-diphenyltetrazolium bromide; Merck). The supernatants were discarded, the cells lysed using dimethyl sulfoxide (Merck), and the optical density measured using two technical replicates at 570 nm (OD_{570}) using a POLARstar Omega plate reader (BMG Labtech, Aylesbury, Buckinghamshire, UK).

Western blotting

Granulosa cells were washed with 300 µl ice-cold PBS and lysed with 100 µl of PhosphoSafe Extraction Reagent (Novagen, Darmstadt, Germany), followed by protein extraction and quantification by DC Assay. Proteins (10 µg) were electrophoresed on a 10% or 12% polyacrylamide gel, transferred onto a PVDF membrane (GE Healthcare), blocked for 1 h in 5% BSA in Tris-buffered saline-Tween 20 (TBS-Tween), and probed overnight with primary antibodies diluted 1:1000 in 5% BSA TBS-Tween. The primary antibodies were SCARB1 (Bio-Techne, Minneapolis, MN, USA; RRID: AB_10107658), LDLR (Bio-Techne; RRID: AB_11016939) or HMGCR (Abcam, Cambridge, UK; RRID: AB_2749818). After three 5 min washes in TBS-Tween, membranes were incubated for 60 min with 1:2500 dilution HRP-linked anti-mouse IgG (Cell Signaling; RRID: AB_330924) or anti-rabbit IgG (Cell

Signaling; RRID: AB_2099233). Following a further three washes, protein reactivity was assessed by enhanced chemiluminescence using Clarity Western ECL substrate (Bio-Rad). After imaging, membranes were stripped for 7 min with Restore Western Blot Stripping Buffer (Fisher Scientific) and re-probed with 1:1000 dilution β-actin (Abcam; RRID: AB_306371) or α-tubulin (Cell Signaling; RRID: AB_2619646), which were used to normalise protein loading. Images of whole blots were captured using a ChemiDoc XRS System (Bio-Rad). The background-normalised peak band density was quantified in the images for each protein using Fiji (Schindelin*, et al.* 2012), with target protein bands normalised to β-actin or α-tubulin.

Measurement of cholesterol in granulosa cells

Granulosa cells were cultured in granulosa cell medium and treated for 24 h with control, 1 μg/ml LPS, or 1 mM methyl-β-cyclodextrin. The cells were then washed twice with PBS, collected in 200 μl/well cholesterol assay buffer (Thermo Fisher Scientific), and total cellular cholesterol concentrations were measured in duplicate using the Amplex Red Cholesterol Assay (Thermo Fisher Scientific), according to the manufacturers' instructions. The inter-assay and intra-assay coefficients of variation were both < 6%. Total cellular cholesterol concentrations were normalized to total cellular protein concentrations, quantified using a DC assay (Bio-Rad), as described previously (Nicholson and Ferreira 2009).

Measurement of HDL and LDL cholesterol in serum and follicular fluid

Using ovaries collected from 15 animals after slaughter, follicular fluid was aspirated from 47 follicles 4 to 16 mm diameter, and the concentration of cholesterol was determined in duplicate using a cholesterol oxidase-endpoint assay (Randox Daytona Plus, Randox Laboratories Ltd, Crumlin, UK), as described previously (Piersanti*, et al.* 2019). In addition, total cholesterol, HDL and LDL/VLDL concentrations were quantified in samples of follicular fluid and serum using LDL/VLDL precipitation buffer (Abcam) and the Amplex Red cholesterol assay, according to the manufacturers' instructions.

Statistical analysis

The statistical unit was each independent culture of granulosa cells, collected on separate days and pooled from the ovaries of 10 to 20 animals. Statistical analysis was performed using GraphPad Prism 8.4.2 (GraphPad Software, San Diego, California, USA). Data are reported as arithmetic mean ± SEM, and significance attributed when $P < 0.05$. Statistical comparisons

between treatments or challenges were made using two-sided independent *t*-test, or using ANOVA followed by Dunnett's or Bonferroni's *post hoc* test for multiple comparisons.

Results

Serum and follicular fluid increase granulosa cell inflammatory responses to LPS

Challenging granulosa cells isolated from either 4–8 mm or > 8.5 mm diameter ovarian follicles with 1 μg/ml LPS stimulated the secretion of IL-1α, IL-1β and IL-8 (all $P < 0.001$, ANOVA, Fig. 1), without significantly affecting cell viability (all $P > 0.6$, t-test). Culturing the granulosa cells from either 4–8 mm diameter or > 8.5 mm diameter ovarian follicles with serum increased the LPS-stimulated secretion of IL-1α and IL-1β but not IL-8 (Fig. 1A).

Granulosa cells are bathed in follicular fluid rather than serum *in vivo*, and follicular fluid differs from serum because of the limited permeability of the follicle basement membrane to LDL and VLDL cholesterol (Siu and Cheng 2012). Culturing granulosa cells with bovine follicular fluid increased LPS-stimulated IL-1α and IL-8 secretion, but not IL-1β secretion (Fig. 1B). The viability of granulosa cells isolated from either 4–8 mm diameter or > 8.5 mm diameter ovarian follicles was not significantly altered by serum or follicular fluid (Fig. 1A, B).

Methyl-β-cyclodextrin reduces granulosa cell inflammatory responses to LPS

To examine whether cholesterol might be important for serum increasing LPS-stimulated inflammation, we used methyl-β-cyclodextrin to bind cholesterol (Christian, *et al.* 1997). Granulosa cells were cultured for 24 h in granulosa cell medium supplemented with 10% serum and treated with a range of concentrations of methyl-β-cyclodextrin, and then challenged for 24 h with LPS in the continued presence of the methyl-β-cyclodextrin. Methyl-β-cyclodextrin reduced LPS-stimulated secretion of IL-1α and IL-1β in granulosa cells from both 4–8 mm and > 8.5 mm diameter ovarian follicles, and reduced the secretion of IL-8 in cells from 4–8 mm but not > 8.5 mm diameter ovarian follicles (Fig. 2). The reduction in inflammation was similar to that of an established anti-inflammatory glucocorticoid, dexamethasone (Cain and Cidlowski 2017). Compared with vehicle, the highest concentration of methyl-β-cyclodextrin did not significantly alter cell viability as determined by the amount of protein per culture well in granulosa cells from 4–8 mm diameter ovarian follicles (2.1 ± 0.36 vs. 1.9 ± 0.33 mg protein, *t*-test, $P = 0.72$, $n = 4$) or > 8.5 mm diameter ovarian follicles (1.5 ± 0.2 vs. 1.2 ± 0.29 mg protein, $P = 0.50$, $n = 4$), or by MTT assays of granulosa cells

from 4–8 mm diameter ovarian follicles (0.9 ± 0.02 vs. 1.0 ± 0.06 OD_{570}, P = 0.07, ANOVA, n = 4) or > 8.5 mm diameter ovarian follicles (0.64 ± 0.1 vs. 0.94 ± 0.09 OD_{570}, P = 0.09, ANOVA, n = 4).

Granulosa cell and follicular fluid cholesterol

We measured the total cellular cholesterol of granulosa cells cultured for 24 h in control serum free medium using the Amplex Red Cholesterol Assay. The total cellular cholesterol was 34.3 ± 4.7 μg/mg protein in granulosa cells from 4–8 mm diameter ovarian follicles, and 35.1 ± 4.6 μg/mg protein in cells from > 8.5 mm diameter ovarian follicles (both n = 5). Culturing granulosa cells from 4–8 mm diameter or > 8.5 mm diameter ovarian follicles for 24 h with 1 μg/ml LPS reduced total cellular cholesterol to 76.5 ± 7.4% and 80.1 ± 6.3% of control, respectively (*t*-test, both n = 5, P < 0.05). As a control, culturing granulosa cells from 4–8 mm diameter or > 8.5 mm diameter ovarian follicles for 24 h with 1 mM methyl-β-cyclodextrin reduced cholesterol to 63.0 ± 6.6% and 60.6 ± 6.4% of control, respectively (*t*-test, both n = 5, P < 0.001).

As cholesterol might be important for inflammatory responses to LPS, we measured the total cholesterol, HDL cholesterol, and LDL/VLDL cholesterol in the fetal bovine serum and bovine follicular fluid used in our cultures. Serum contained 851 ± 116 μg/ml total cholesterol (n = 3 samples), and follicular fluid contained 580 ± 38 μg/ml cholesterol (n = 47 follicles from 15 animals). There was more HDL than LDL/VLDL cholesterol in serum (152 ± 7 vs 71 ± 7 μg/ml, n = 3 samples, *t*-test P < 0.001), and in follicular fluid from 4–8 mm diameter ovarian follicles (322 ± 30 vs 41 ± 13 μg/ml, n = 3 animals, *t*-test P < 0.001) and from > 8.5 mm diameter ovarian follicles (307 ± 31 vs 29 ± 9 μg/ml, n = 3 animals, *t*-test P < 0.001).

We next examined whether granulosa cells expressed receptors for cholesterol using Western blotting. Granulosa cells isolated from 4–8 mm diameter and > 8.5 mm diameter ovarian follicles expressed SCARB1 and the 120 kDa LDLR precursor protein (Fig. 3). However, the 160 kDa mature form of LDLR protein was barely detectable when analysed by Western blot. We concluded that granulosa cells are exposed to more HDL than LDL/VLDL, and expressed SCARB1, which is the receptor for HDL.

HDL cholesterol increases granulosa cell inflammatory responses to LPS

As serum and follicular fluid contained HDL, LDL and VLDL cholesterol, we examined inflammatory responses to LPS when granulosa cells were cultured with each of these sources

of cholesterol. Culturing cells with HDL cholesterol increased the LPS-stimulated secretion of IL-1α and IL-1β, but not IL-8, in granulosa cells (Fig. 4A). However, there was no significant alteration in LPS-stimulated secretion of IL-1α, IL-1β or IL-8 when granulosa cells were cultured with LDL cholesterol (Fig. 4B) or with VLDL cholesterol (Supplementary Figure 2). Cell viability was not significantly altered by treatment with HDL cholesterol (all values within 87% of control; 4–8 mm diameter, ANOVA $P = 0.83$, $n = 5$; > 8.5 mm diameter, $P = 0.62$, $n = 5$), LDL cholesterol (all values within 85% of control; 4–8 mm diameter, ANOVA $P = 0.97$, $n = 3$; > 8.5 mm diameter, $P = 0.86$, $n = 2$) or VLDL cholesterol (all values within 83% of control; 4–8 mm diameter, ANOVA $P = 0.93$, $n = 3$; > 8.5 mm diameter, $P = 0.97$, $n = 2$). Collectively, the use of cholesterol by granulosa cells, the increased LPS-stimulated inflammatory response with HDL cholesterol, the expression of SCARB1 protein by granulosa cells, and the abundant HDL cholesterol in follicular fluid, imply that HDL cholesterol supports granulosa cell inflammatory responses to LPS.

HDL cholesterol and serum do not alter oestradiol or progesterone secretion from granulosa cells

One concern was that the effects of HDL cholesterol or serum on LPS-stimulated inflammatory responses might be mediated by changes in steroidogenesis, even in the absence of androstenedione or gonadotrophins. However, treating granulosa cells isolated from > 8.5 mm diameter ovarian follicles with 100 μg/ml HDL or 10% serum for 24 h, followed by a 24 h challenge with control medium or LPS, did not significantly alter the secretion of oestradiol or progesterone compared with vehicle ($P = 0.77$, ANOVA, Fig. 5). There was also no significant effect of LPS challenge on the secretion of oestradiol or progesterone compared with control ($P = 0.89$, ANOVA, Fig. 5). Treatment with 50 μg/ml LDL for 48 h also did not significantly alter the secretion of oestradiol or progesterone compared with vehicle ($P = 0.88$ and $P = 0.43$, respectively, t-test, Supplementary Figure 3).

Cholesterol biosynthesis has a role in granulosa cell inflammatory responses to LPS

As well as receptor-mediated cholesterol uptake, eukaryotic cells synthesise cholesterol via the mevalonate pathway, which is regulated by the HMGCR enzyme (Goldstein and Brown 1990). The first committed step of cholesterol synthesis depends on farnesyl diphosphate (generated by the mevalonate pathway enzyme FDPS) being converted to squalene by FDFT1. To explore whether cholesterol biosynthesis might also support inflammatory responses to LPS, we used previously validated siRNA targeting *HMGCR*, *FDPS* and *FDFT1*

(Griffin*, et al.* 2018, Griffin*, et al.* 2017, Healey*, et al.* 2016). We verified that transfecting granulosa cells pooled from 4–8 mm and > 8.5 mm diameter ovarian follicles with siRNA for 48 h, reduced the expression of *HMGCR*, *FDPS* and *FDFT1* by 56%, 67% and 55% compared with scramble siRNA, respectively (Supplementary Figure 4). Transfecting granulosa cells with siRNA targeting *HMGCR*, *FDPS* or *FDFT1* for 24 h, followed by a 24 h challenge with control medium or LPS reduced the LPS-stimulated secretion of IL-1α, IL-1β and IL-8 by granulosa cells (Fig. 6A). The targeting siRNA did not significantly alter granulosa cell viability compared with scramble siRNA (4–8 mm diameter, all values within 92% of scramble, ANOVA $P = 0.84$, $n = 5$; > 8.5 mm diameter, all values within 96% of scramble, $P = 0.26$, $n = 3$). In addition, transfecting granulosa cells with siRNA targeting *HMGCR*, *FDPS* or *FDFT1* for 48 h did not significantly alter SCARB1 protein in granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles (Fig. 6B). Collectively, these findings imply that cholesterol biosynthesis also plays a role in supporting LPS-stimulated inflammation in granulosa cells.

FSH increases granulosa cell inflammatory responses to LPS

We finally considered the role of FSH in granulosa cell inflammatory responses to LPS, because FSH could stimulate cholesterol biosynthesis or SCARB1 uptake of HDL (Bertevello*, et al.* 2018, Lai*, et al.* 2013, Reverchon*, et al.* 2014). Under serum-free conditions, treatment for 24 h with a supraphysiological concentration of 2.5 μg/ml FSH, with or without 10^{-7} M androstenedione, increased LPS-stimulated secretion of IL-1α, IL-1β and IL-8 in granulosa cells from 4–8 mm diameter ovarian follicles, and the secretion of IL-1α and IL-1β in granulosa cells from > 8.5 mm diameter ovarian follicles (Fig. 7A). However, the same treatments in granulosa culture medium supplemented with 10% serum had little effect on LPS-stimulated inflammation in granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles (Supplementary Figure 5). Notably, supplying androstenedione alone, with or without serum, did not significantly alter LPS-stimulated inflammation.

To examine whether gonadotropin-augmented increases in inflammation extended to LH, we used a range of concentrations of FSH and LH to treat granulosa cells, which were isolated from > 8.5 mm diameter ovarian follicles because they express both FSH and LH receptors (Herath*, et al.* 2007, Xu*, et al.* 1995). Under serum-free conditions, treatment with supraphysiological concentrations of FSH increased LPS-stimulated secretion of IL-1α, IL-1β

and IL-8 (Fig. 7B). However, a range of concentrations of LH did not significantly alter LPS-stimulated secretion of IL-1α, IL-1β or IL-8 (Fig. 7C).

We also examined whether the FSH-augmented increases in LPS-stimulated inflammation were associated with altered steroidogenesis. Under serum-free conditions, treating granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles with 10^{-7} M androstenedione increased the accumulation of oestradiol but not progesterone (Fig. 8). However, treatment with 2.5 μg/ml FSH, or FSH and androstenedione, did not significantly alter oestradiol secretion. Treatment with androstenedione and FSH, but not androstenedione or FSH alone, increased the accumulation of progesterone. The LPS challenge reduced the androstenedione-augmented increase in oestradiol by granulosa cells from 4–8 mm but not > 8.5 mm diameter ovarian follicles (Fig. 8). In the presence of 10% serum, treatment of granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles with androstenedione also increased the accumulation of oestradiol but not progesterone, and FSH did not significantly alter oestradiol or progesterone accumulation (Supplemental Figure 6). Taken together, the effects of FSH on LPS-stimulated inflammation did not appear to be associated with altered steroidogenesis in granulosa cells.

Finally, we examined whether FSH treatment altered granulosa cell SCARB1 or HMGCR protein abundance. Treatment with 2.5 μg/ml FSH but not 10^{-7} M androstenedione, increased SCARB1 protein in granulosa cells from 4–8 mm and > 8.5 mm diameter ovarian follicles cultured in serum-free medium, or granulosa culture medium supplemented with 2% but not 10% serum (Fig. 9). However, treatment with FSH had little effect on HMGCR protein abundance (Fig. 9). Together, these observations provide evidence that FSH increases SCARB1 protein in granulosa cells and that FSH increases inflammatory responses to LPS.

Discussion

It was previously reported that granulosa cells have limited responses to LPS under serum-free conditions (Bromfield and Sheldon 2011). Here, we found that serum or follicular fluid increased LPS-stimulated secretion of IL-1α and IL-1β from granulosa cells isolated from 4–8 mm diameter and > 8.5 mm diameter ovarian follicles. Conversely, depleting cholesterol using methyl-β-cyclodextrin diminished LPS-stimulated granulosa cell secretion of IL-1α and IL-1β. Follicular fluid contained abundant HDL cholesterol, and granulosa cells expressed the HDL receptor SCARB1. Furthermore, supplying HDL cholesterol increased LPS-stimulated inflammation in granulosa cells. As well as receptor-mediated uptake, cholesterol

biosynthesis might also play a role in LPS-stimulated inflammation because RNA interference of mevalonate pathway enzymes inhibited inflammation. Treatment with supraphysiological concentrations of FSH, but not LH, also increased LPS-stimulated granulosa cell inflammation, and increased SCARB1 protein. However, changes in inflammation were not associated with changes in oestradiol or progesterone secretion. Taken together these findings imply that cholesterol supports granulosa cell inflammatory responses to LPS.

Our key finding was that serum, follicular fluid, and HDL cholesterol increased LPS-stimulated inflammation in granulosa cells. The increased inflammatory response to LPS in the presence of HDL cholesterol, and the lack of response with LDL or VLDL cholesterol, also reflected the far greater abundance of HDL than LDL/VLDL cholesterol in follicular fluid. The 100 μg/ml concentration of HDL that increased inflammatory responses to LPS was less than the concentration of HDL cholesterol in follicular fluid, whilst the lack of response to LPS with up to 50 μg/ml LDL or 10 μg/ml VLDL cholesterol exceeded their concentrations in follicular fluid. The difference in lipoprotein abundance in follicular fluid is thought to be because the follicle basement membrane only allows diffusion of HDL, and any LDL found in follicular fluid is thought to be synthesised by the granulosa cells (Dunning*, et al.* 2014, Siu and Cheng 2012). Commensurate with abundant HDL cholesterol in follicular fluid, we found that granulosa cells expressed SCARB1. Only the precursor form of the LDL receptor was detectable in the present study, and precursor LDLR requires glycosylation to form mature LDLR (Cummings*, et al.* 1983). Therefore, we suggest that HDL cholesterol may have a role in supporting inflammatory response to LPS in granulosa cells.

Granulosa cell inflammatory responses to LPS depended on cholesterol biosynthesis as well as the supply of cholesterol in culture medium. We found that siRNA targeting *HMGCR*, *FDPS* or *FDFT1* encoding enzymes in cholesterol biosynthesis impaired the inflammatory responses to LPS in granulosa cells. Interestingly, the presence of serum in culture medium did not compensate for the siRNA targeting the cholesterol biosynthesis enzymes. This may be because the regulation of cellular cholesterol depends on the integration of multiple systems, including receptor-mediated cholesterol uptake, cholesterol biosynthesis, cholesterol efflux pathways, isoprenoids, oxysterols, liver x receptors, SREBPs, and aster proteins (Acton*, et al.* 1996, Brown and Goldstein 1976, Kutyavin and Chawla 2018). Furthermore, integration of cholesterol regulation is unusual in granulosa cells, where steroidogenesis is

dominated by aromatization of androstenedione from thecal cells (Bertevello, *et al.* 2018, Dorrington, *et al.* 1975, Liu and Hsueh 1986). This steroidogenesis is independent of HDL cholesterol, presumably to evade cholesterol homeostatic feedback altering steroidogenesis (Bao, *et al.* 1995, Lai, *et al.* 2013, O'Shaughnessy, *et al.* 1990).

The most consistent changes in LPS-stimulated inflammation associated with cholesterol were the increased IL-1α and IL-1β secretion from granulosa cells. Supplying cholesterol or stimulating cholesterol biosynthesis also promotes IL-1β secretion from LPS-stimulated bone-marrow-derived macrophages, and this was associated with altered mitochondrial function (Dang, *et al.* 2017). Whereas, treating human monocytes with methyl-β-cyclodextrin limited inflammatory responses to LPS (Triantafilou, *et al.* 2002). We also found that treating granulosa cells with methyl-β-cyclodextrin diminished LPS-stimulated inflammation. One mechanism linking cholesterol to inflammation is that TLR4 and CD14 are located in cholesterol-rich microdomains in plasma membranes, and increased fluidity of membranes may facilitate the interaction of TLR4 and CD14 for sensing LPS and mounting inflammatory responses (Triantafilou, *et al.* 2002). Although we verified that methyl-β-cyclodextrin reduced cellular cholesterol, methyl-β-cyclodextrin can have other direct or indirect effects on cells (Zidovetzki and Levitan 2007). Therefore, further work will be needed to determine how cholesterol is mechanistically linked to inflammation in granulosa cells. Another question worth investigating is whether the reduced availability of cholesterol during postpartum metabolic stress may diminish ovarian follicle immunity.

We considered the role of FSH in granulosa cell responses to LPS because FSH can stimulate cholesterol biosynthesis and the uptake of HDL cholesterol (Bertevello, *et al.* 2018, Lai, *et al.* 2013, Reverchon, *et al.* 2014). We found that supraphysiological concentrations of FSH increased SCARB1 protein and enhanced inflammatory responses to LPS in granulosa cells under serum-free conditions. These observations are similar to findings with murine granulosa cells, where 100 ng/ml FSH increased SCARB1 protein, and enhanced inflammatory responses to LPS (Shimada, *et al.* 2006). It is thought that FSH priming of innate immunity in granulosa cells may have roles in defence against pathogens, and in the inflammatory response to endogenous ligands during ovulation and fertilization. One possible mechanism is that scavenger receptors such as SCARB1 can act as co-receptors, presenting LPS to TLR4 (Vasquez, *et al.* 2017). For example, LPS binds to CLA-1, a human SCARB1 analogue, and is internalized independently or in association with HDL (Vishnyakova, *et al.*

2003). Furthermore, LPS stimulation of transgenic mice overexpressing SCARB1 had 2 to 3-fold higher concentrations of serum cytokine concentrations than wild-type mice (Baranova, *et al.* 2016). Future experiments might attempt to disentangle the relative roles for SCARB1 in immunity, steroidogenesis, and cell replication.

A limitation of the present study was that granulosa cells spontaneously luteinize during culture, particularly when cultured with serum and at high seeding densities (Glister, *et al.* 2001, Gutierrez, *et al.* 1997, Portela, *et al.* 2010, Spicer, *et al.* 2002). Furthermore, serum diminishes the responsiveness of bovine granulosa cells to FSH during culture (Gong, *et al.* 1993, Gutierrez, *et al.* 1997). However, granulosa cells do not respond to LPS in serum-free conditions and treatment with 2.5 μg/ml FSH in medium containing 10% serum was needed to increase the LPS-induced secretion of IL-6 and IL-8 (Bromfield and Sheldon 2011). We acknowledge that the granulosa cells in the current study displayed signs of luteinization because the progesterone concentrations were higher than the oestradiol concentrations. Although, in the present study, granulosa cell inflammatory responses to LPS were still stimulated by supraphysiological concentrations of FSH. Culturing granulosa cells has provided important knowledge about granulosa cell physiology and pathology over the last 50 years. Whether granulosa cells should be cultured with FSH, serum, follicular fluid, or using synthetic supplements in serum-free media depends on the biological question being studied (Gutierrez, *et al.* 1997). For example, follicular fluid and serum both contain HDL cholesterol, but differ in their LDL/VLDL cholesterol content and other respects, including the presence of hormones. Our findings imply that HDL cholesterol might be considered as a supplement in serum-free media for culturing granulosa cells to study inflammation.

The response to LPS and the importance of cholesterol for inflammation was similar for granulosa cells from both 4–8 mm diameter and > 8.5 mm diameter ovarian follicles. These observations were initially surprising because the follicle sizes were selected to represent cells from physiologically different emerged and dominant follicles, respectively (Fortune 1994). Granulosa cells from > 8.5 mm diameter dominant follicles secrete more oestradiol than granulosa cells from emerged follicles, and express LH as well as FSH receptors (Gutierrez, *et al.* 1997, Herath, *et al.* 2007, Xu, *et al.* 1995). However, the findings in the present study agree with previous observations that granulosa cell inflammatory responses to LPS are independent of follicle development (Bromfield and Sheldon 2011, Horlock, *et al.* 2021, Price, *et al.* 2013). In the present study, the effects of serum, HDL cholesterol, and FSH on

LPS-stimulated inflammation did not appear to be associated with changes in oestradiol or progesterone accumulation. Furthermore, we previously found that treating granulosa cells with exogenous oestradiol or progesterone did not significantly alter the IL-6 response to LPS (Price and Sheldon 2013). Taken together these findings imply that the evolutionary ancient roles of innate immunity and cholesterol metabolism may eclipse physiological changes in granulosa cells.

In conclusion, we found evidence that supplying cholesterol increased LPS-stimulated inflammation in bovine granulosa cells. Linking cholesterol with LPS-stimulated inflammation in granulosa cells is an example of immunometabolism - the integration of immunity and metabolism (O'Neill, *et al.* 2016). Interestingly, both HDL cholesterol and cholesterol biosynthesis contributed to inflammatory responses. Our findings imply that cholesterol supports granulosa cell inflammatory responses to LPS.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

Conceptualization IMS, JJB, ADH; methodology ADH, IMS; investigation ADH; formal analysis ADH, IMS; writing - original draft preparation ADH, IMS; writing - review and editing IMS, ADH, TJRO, MJDC, JJB, JEPS; visualization ADH, IMS; supervision IMS, MJDC, JJB; project administration IMS, JJB; funding acquisition JJB, IMS, JEPS.

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Figure Legends

Figure 1. Serum and follicular fluid increase granulosa cell inflammatory responses to LPS

Granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles were cultured for 24 h with the indicated percentage of (A) fetal bovine serum or (B) follicular fluid, and then challenged for 24 h with control medium (●) or 1 µg/ml LPS (○). Cell supernatant IL-1α, IL-1β and IL-8 concentrations were measured by ELISA, and cell viability assessed by MTT assay. Data are presented as mean + SEM from 3 independent experiments. Statistical significance was determined for the effect of serum or follicular fluid on the LPS response using ANOVA with Dunnett's *post hoc* test; values differ from LPS challenge without serum or follicular fluid, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2. Methyl-β-cyclodextrin reduces granulosa cells inflammatory responses to LPS

Granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles were treated for 24 h with the indicated concentrations of methyl-β-cyclodextrin (MβCD), or 1 µM dexamethasone (Dex), and then challenged for 24 h with control medium (●) or 1 µg/ml LPS (○) in the continued presence of the treatments. Cell supernatant IL-1α, IL-1β and IL-8 concentrations were measured by ELISA. Data are presented as mean + SEM from 4 independent experiments. Statistical significance was determined for the effect of MβCD on the LPS response using ANOVA with Dunnett's *post hoc* test; values differ from LPS challenge without MβCD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3. Granulosa cells express SCARB1

Granulosa cells from 4–8 mm and > 8.5 mm diameter ovarian follicles were cultured for 48 h in granulosa cell medium containing 10% serum, and SCARB1 and LDLR protein analysed by Western blot. Representative Western blots of SCARB1, LDLR and α-tubulin are shown for samples from 4 independent experiments.

Figure 4. HDL cholesterol increases granulosa cell IL-1 responses to LPS

Granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles were treated for 24 h with the indicated concentrations of (A) HDL or (B) LDL cholesterol and then challenged for 24 h with control medium (●) or 1 µg/ml LPS (○), in the continued presence of the treatments. Cell supernatant IL-1α, IL-1β or IL-8 concentrations were measured by ELISA.

Data are presented as mean + SEM from ≥ 3 independent experiments. Statistical significance was determined for the effect of HDL or LDL on the LPS response using two-way ANOVA with Dunnett's *post hoc* test; values differ from LPS challenge without HDL or LDL, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5. HDL cholesterol and serum do not alter oestradiol or progesterone secretion from granulosa cells

Granulosa cells from > 8.5 mm diameter ovarian follicles were treated for 24 h in granulosa cell culture medium containing either vehicle, 100 µg/ml HDL cholesterol or 10% fetal bovine serum, and then challenged for a further 24 h with control medium (●) or medium containing 1 µg/ml LPS (○), in the continued presence of the treatments. Cell supernatant oestradiol and progesterone concentrations were measured by ELISA. Data are presented as mean + SEM from 3 independent experiments.

Figure 6. siRNA targeting mevalonate pathway enzymes reduces granulosa cell inflammatory responses to LPS

(A) Granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles were transfected with scramble siRNA or siRNA targeting *HMGCR*, *FDPS* or *FDFT1* for 24 h, and then challenged for 24 h with control medium (●) or 1 µg/ml LPS (○). Cell supernatant IL-1α, IL-1β and IL-8 concentrations were measured by ELISA. Data are presented as mean + SEM from ≥ 4 independent experiments. Statistical significance was determined using two-way ANOVA with Dunnett's *post hoc* test; values differ from LPS challenge transfected with scramble siRNA, *** $P < 0.001$. (B) Granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles were transfected for 48 h with scramble siRNA or siRNA targeting *HMGCR*, *FDPS* or *FDFT1*, and SCARB1 protein analysed by Western blot. Representative Western blots of SCARB1 and β-actin are shown from 3 independent experiments. Densitometry data were normalized to the β-actin loading control, and presented as mean + SEM.

Figure 7. FSH increases inflammatory responses to LPS in the absence of serum

(A) Granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles were treated in granulosa cell culture medium for 24 h with vehicle, 10^{-7} M androstenedione (A4), 2.5 µg/ml FSH, or androstenedione and FSH, and then challenged for 24 h with control medium (●) or 1 µg/ml LPS (○) in the continued presence of the treatments. Cell supernatant IL-1α, IL-1β and IL-8 concentrations were measured by ELISA. Data are presented as mean + SEM from 4

independent experiments. Statistical significance was determined using two-way ANOVA with Dunnett's *post hoc* test; values differ from LPS challenge treated with vehicle, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B, C) Granulosa cells from > 8.5 mm diameter ovarian follicles were treated for 24 h with the indicated concentrations of FSH (B) or LH (C), and then challenged for 24 h with control medium (●) or 1 µg/ml LPS (○) in the continued presence of the treatments. Cell supernatant IL-1α, IL-1β and IL-8 concentrations were measured by ELISA. Data are presented as mean + SEM from ≥ 3 independent experiments. Statistical significance was determined for the effect of FSH or LH on the LPS response using ANOVA with Dunnett's *post hoc*; values differ from LPS challenge without FSH or LH, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 8. Androstenedione increases oestradiol secretion from granulosa cells

Granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles were treated in granulosa cell culture medium for 24 h with vehicle, 10^{-7} M androstenedione (A4), 2.5 µg/ml FSH, or androstenedione and FSH, and then challenged for 24 h with control medium (●) or 1 µg/ml LPS (○) in the continued presence of the treatments. Cell supernatant oestradiol or progesterone concentrations were measured by ELISA. Data are presented as mean + SEM from 3 independent experiments. Statistical significance was determined using two-way ANOVA with Bonferroni's *post hoc* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 9. FSH increases SCARB1 protein in granulosa cells

Granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles were cultured in granulosa cell culture medium containing 0%, 2% or 10% serum, and treated for 48 h with vehicle, 10^{-7} M androstenedione (A4), 2.5 µg/ml FSH, or androstenedione and FSH, and the cells collected to analyse HMGCR and SCARB1 protein abundance. Representative Western blots of HMGCR, SCARB1 and β-actin are shown from 3 independent experiments. Densitometry data for HMGCR and SCARB1, normalized to the β-actin loading control, are presented as mean + SEM. Statistical significance was determined for the effect of treatment on the LPS response using two-way ANOVA with Dunnett's *post hoc* test; values differ with vehicle, within each serum concentration group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 1

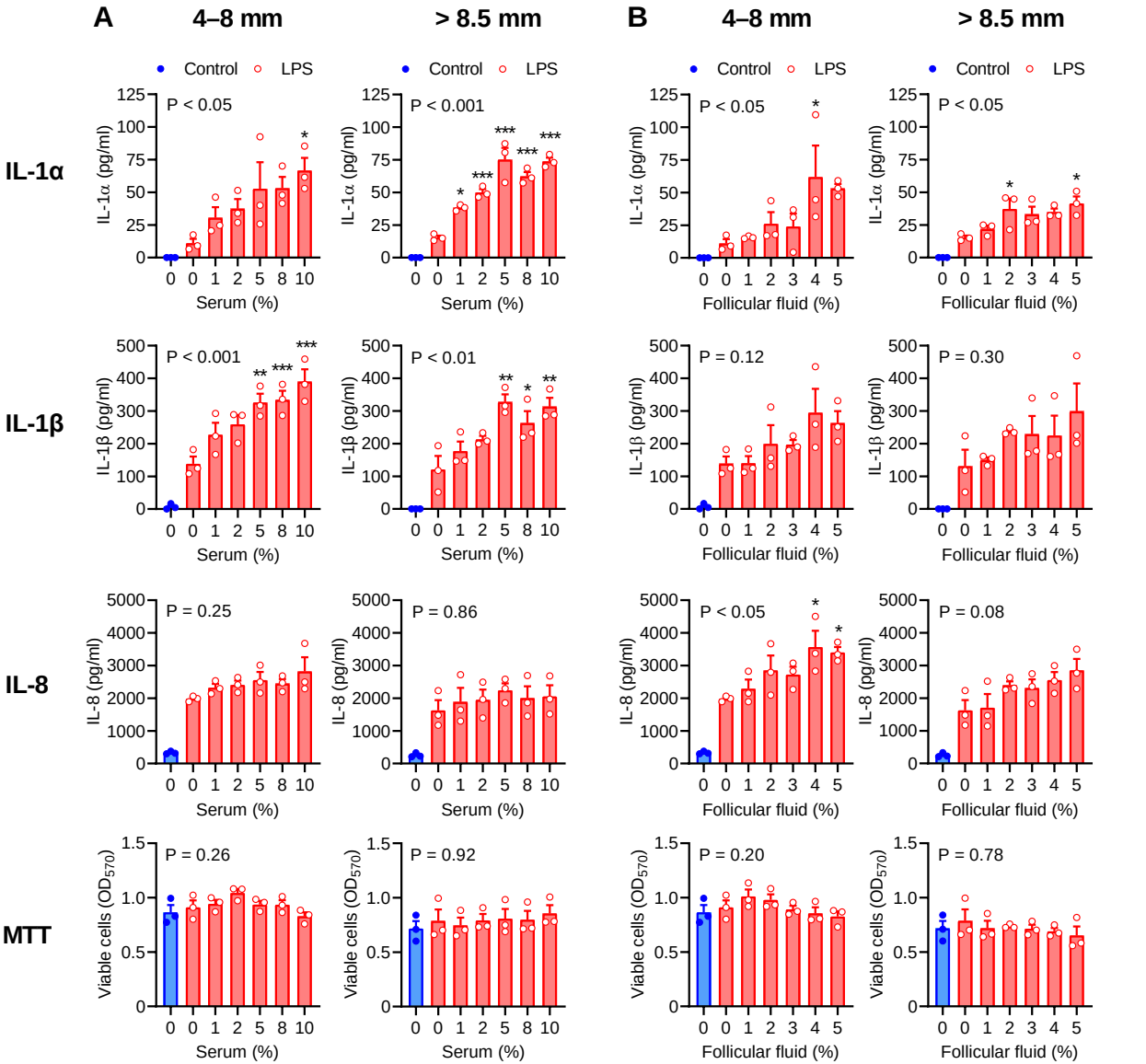

A
4–8 mm
> 8.5 mm
B
4–8 mm
> 8.5 mm
Control
LPS
IL-1α
IL-1β
IL-8
MTT
IL-1α (pg/ml)
IL-1β (pg/ml)
IL-8 (pg/ml)
Viable cells (OD570)
Serum (%)
Follicular fluid (%)
P < 0.05
P < 0.001
P < 0.01
P = 0.25
P = 0.86
P = 0.12
P = 0.30
P = 0.26
P = 0.92
P = 0.20
P = 0.78

Figure 2

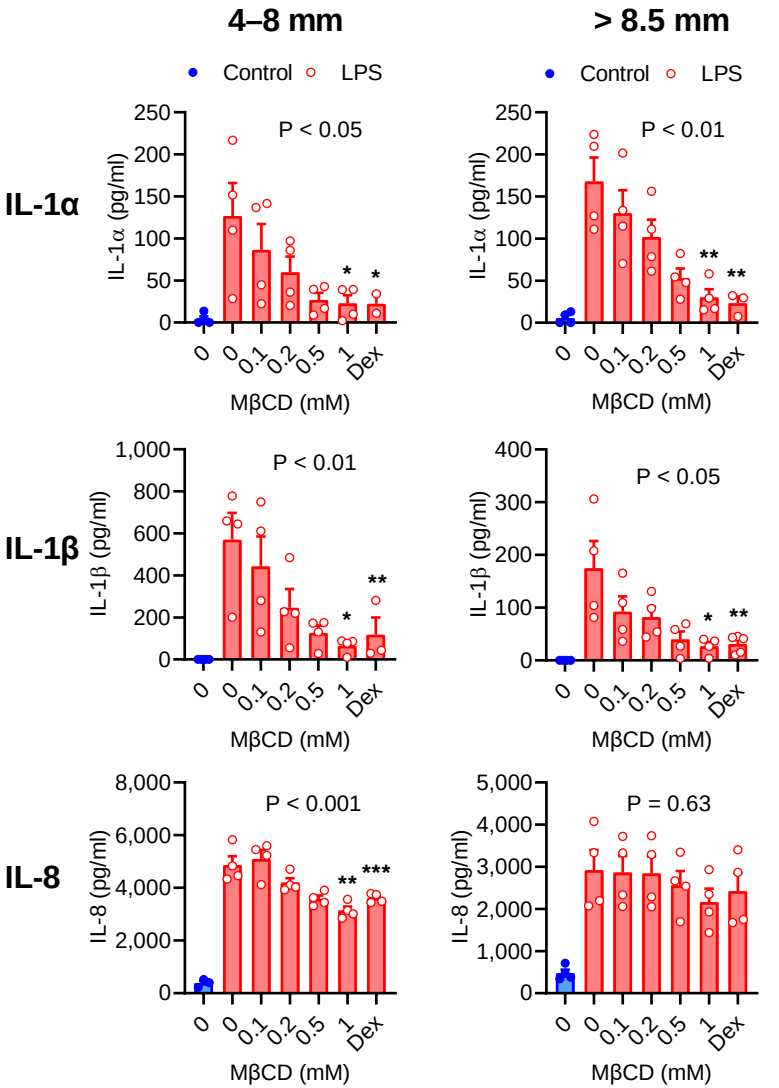

4–8 mm
> 8.5 mm
Control
LPS
IL-1α
IL-1β
IL-8
IL-1α (pg/ml)
IL-1β (pg/ml)
IL-8 (pg/ml)
P < 0.05
P < 0.01
P < 0.001
P = 0.63
MβCD (mM)
0
0
0.1
0.2
0.5
1
Dex
*
**
***

Figure 3

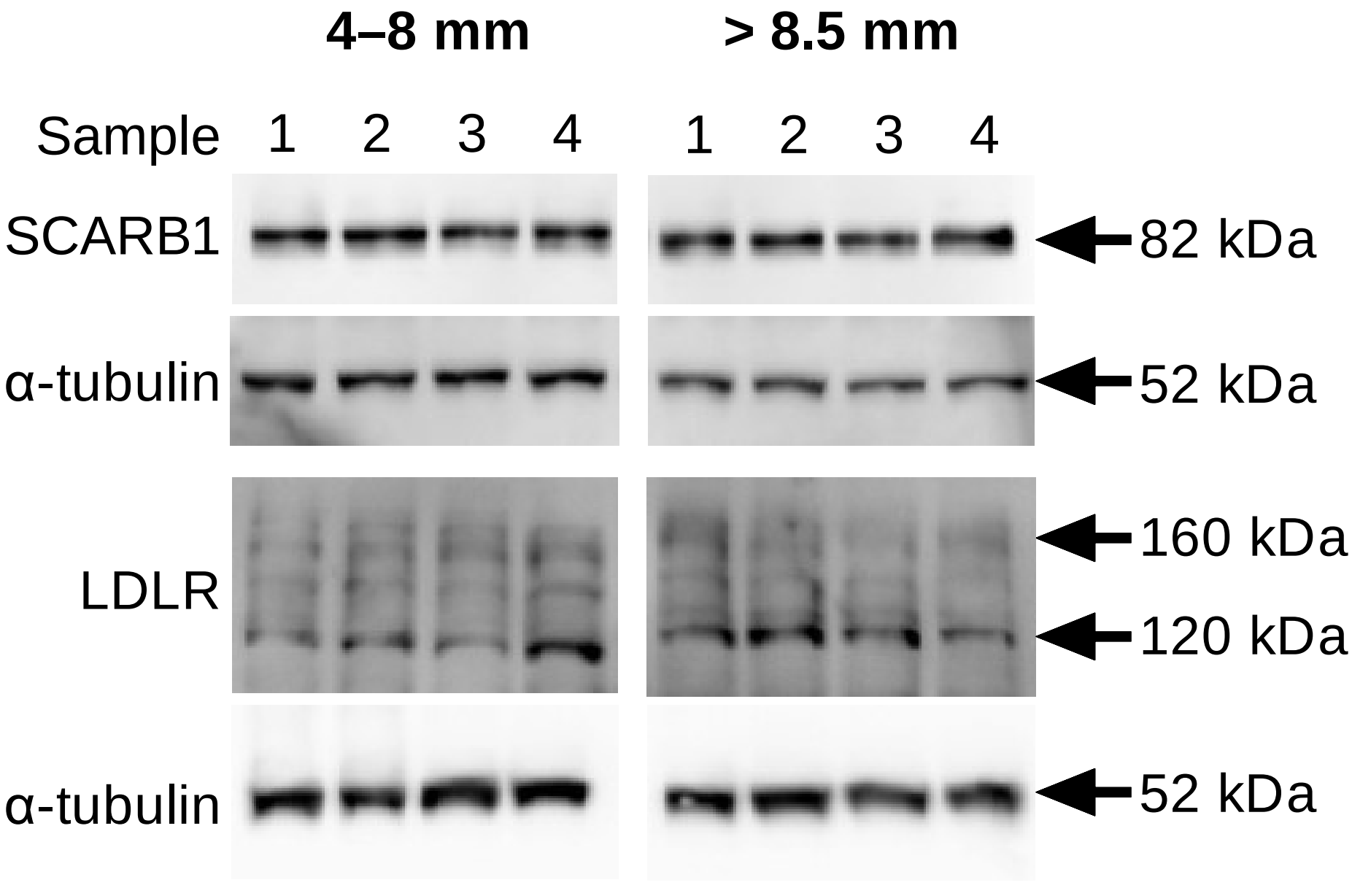

4–8 mm
> 8.5 mm
Sample
1
2
3
4
1
2
3
4
SCARB1
82 kDa
α-tubulin
52 kDa
LDLR
160 kDa
120 kDa
α-tubulin
52 kDa

Figure 4

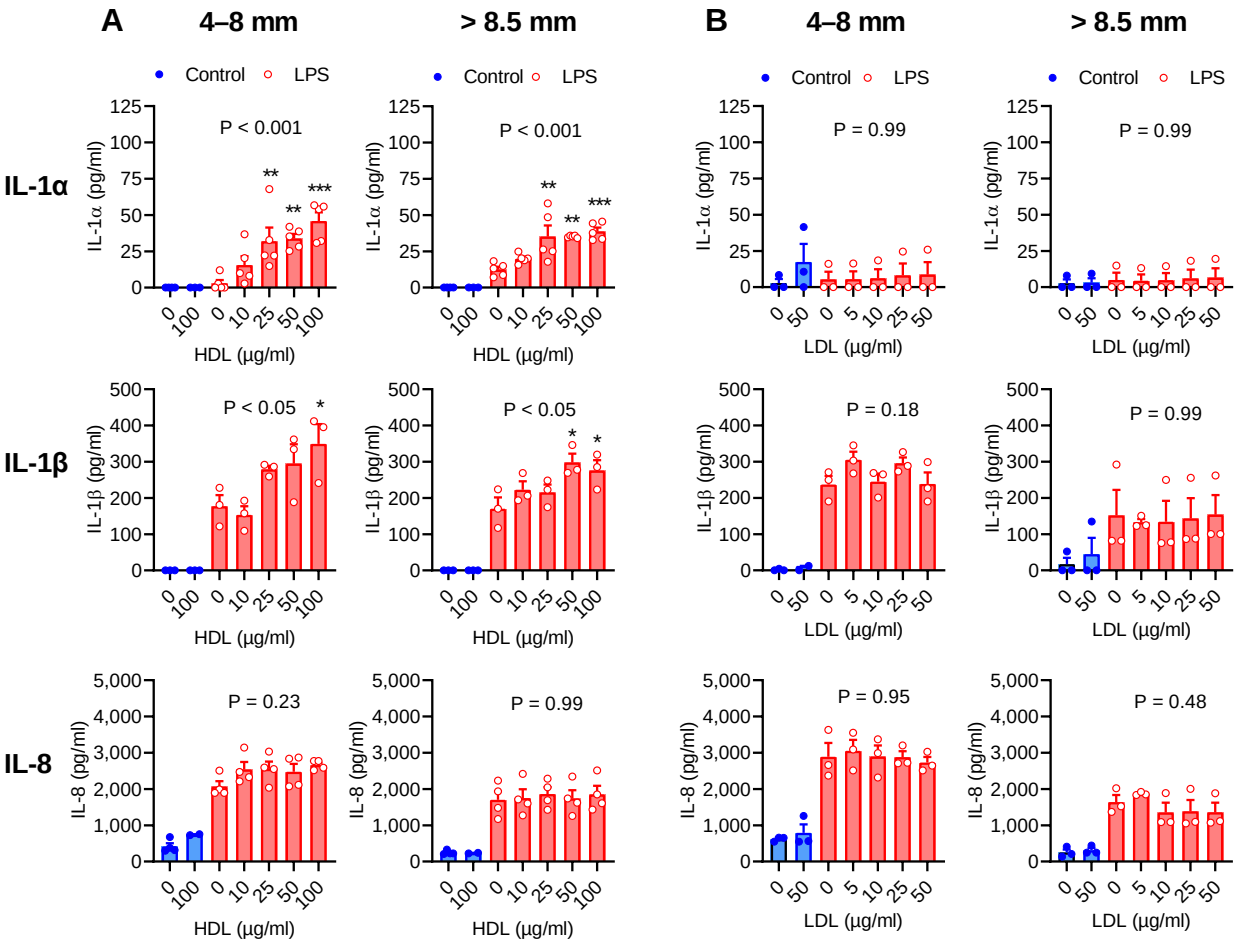
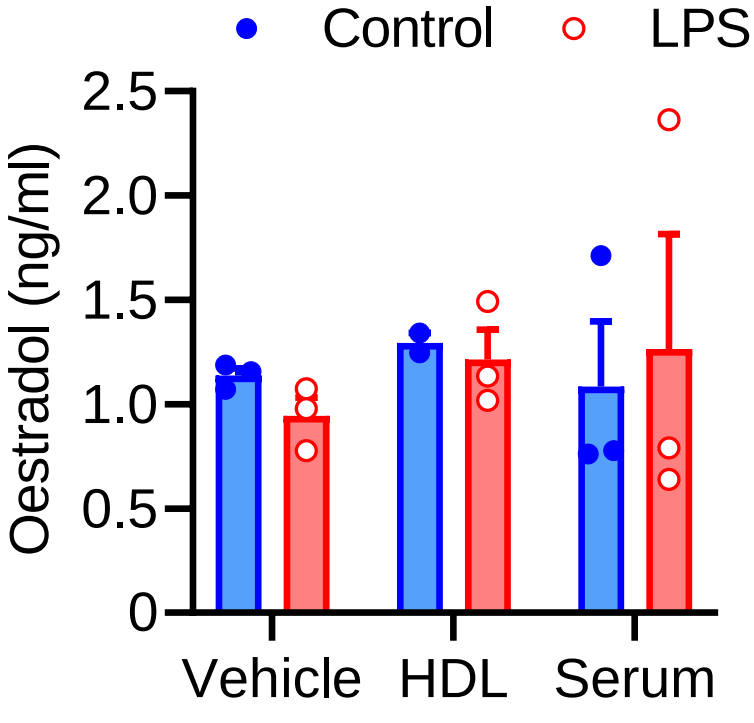

A
4–8 mm
> 8.5 mm
B
4–8 mm
> 8.5 mm
Control
LPS
IL-1α
IL-1β
IL-8
IL-1α (pg/ml)
IL-1β (pg/ml)
IL-8 (pg/ml)
HDL (μg/ml)
LDL (μg/ml)
P < 0.001
P < 0.05
P = 0.23
P = 0.99
P = 0.18
P = 0.95
P = 0.48

Figure 5


Control
LPS
Oestradol (ng/ml)
2.5
2.0
1.5
1.0
0.5
0
Vehicle
HDL
Serum

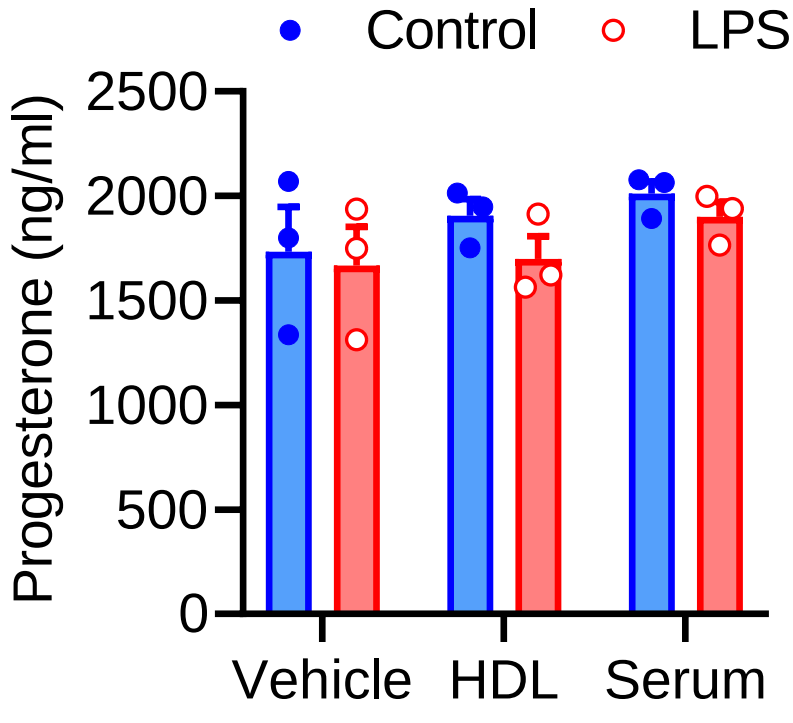

Control
LPS
Progesterone (ng/ml)
2500
2000
1500
1000
500
0
Vehicle
HDL
Serum

Figure 6

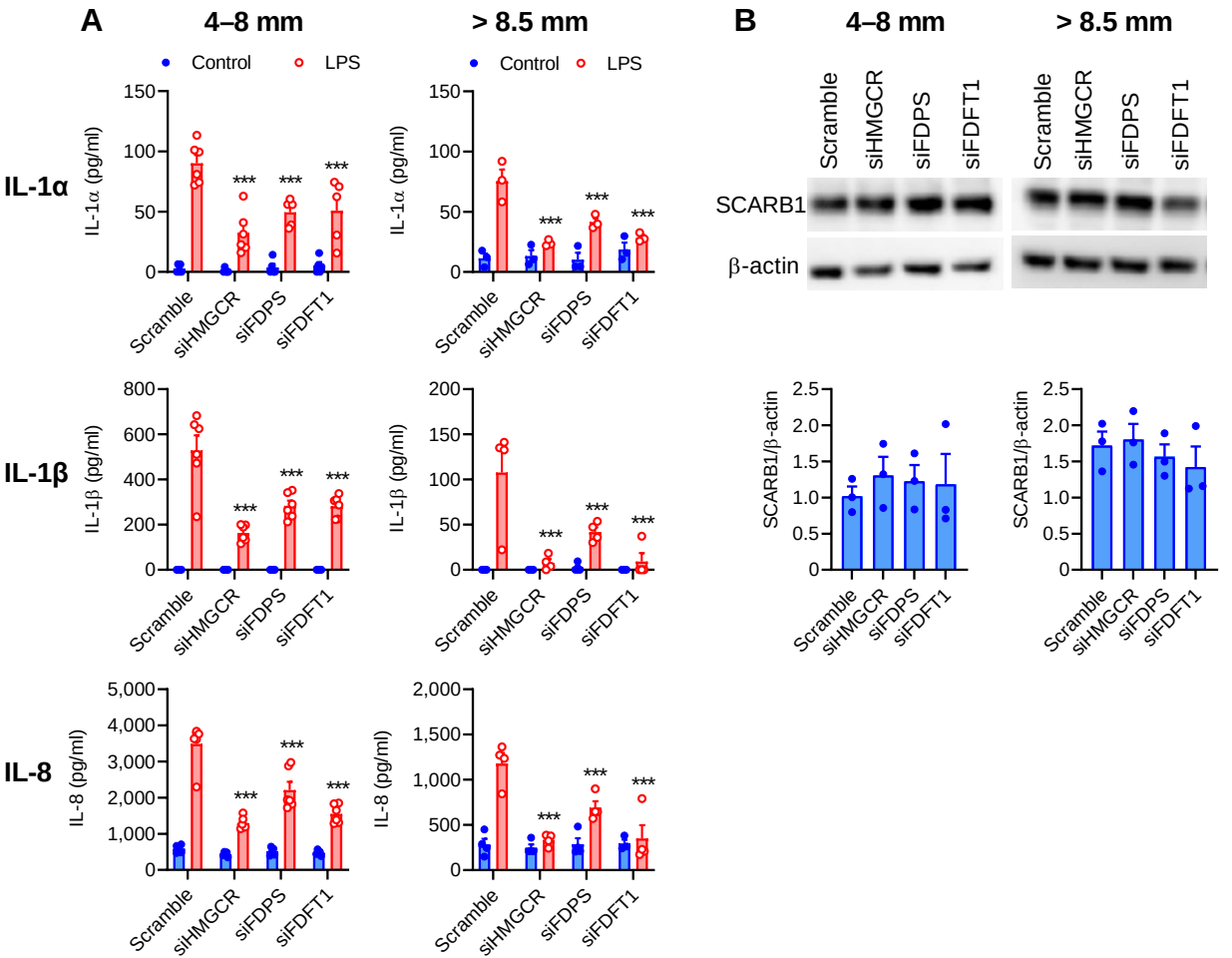

A
4–8 mm
> 8.5 mm
Control
LPS
IL-1α
IL-1β
IL-8
IL-1α (pg/ml)
IL-1β (pg/ml)
IL-8 (pg/ml)
***
Scramble
siHMGCR
siFDPS
siFDFT1
B
4–8 mm
> 8.5 mm
SCARB1
β-actin
SCARB1/β-actin

Figure 7

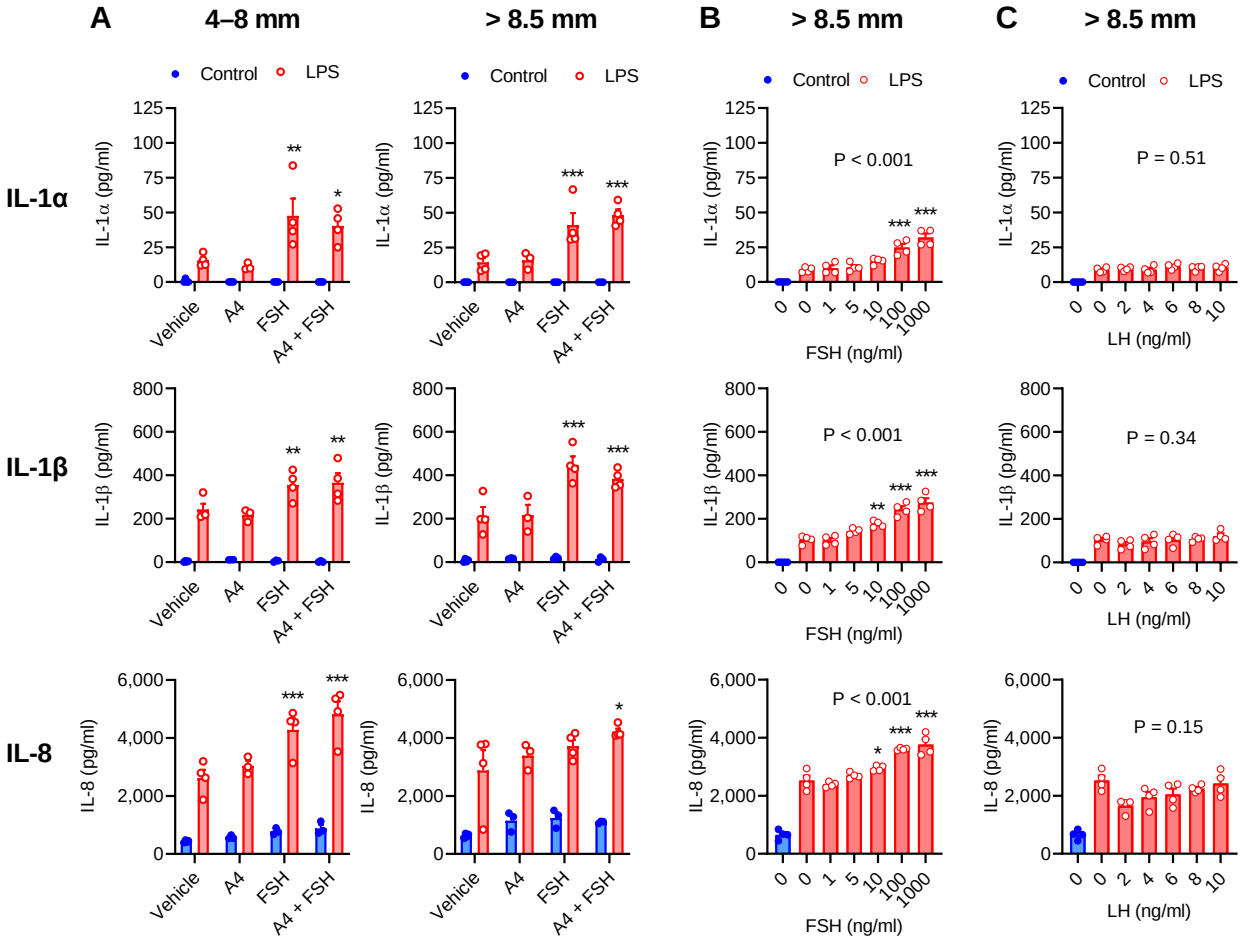

A
4–8 mm
> 8.5 mm
B
> 8.5 mm
C
> 8.5 mm
Control
LPS
IL-1α
IL-1β
IL-8
IL-1α (pg/ml)
IL-1β (pg/ml)
IL-8 (pg/ml)
Vehicle
A4
FSH
A4 + FSH
P < 0.001
P = 0.51
P < 0.001
P = 0.34
P < 0.001
P = 0.15
FSH (ng/ml)
LH (ng/ml)

Figure 8

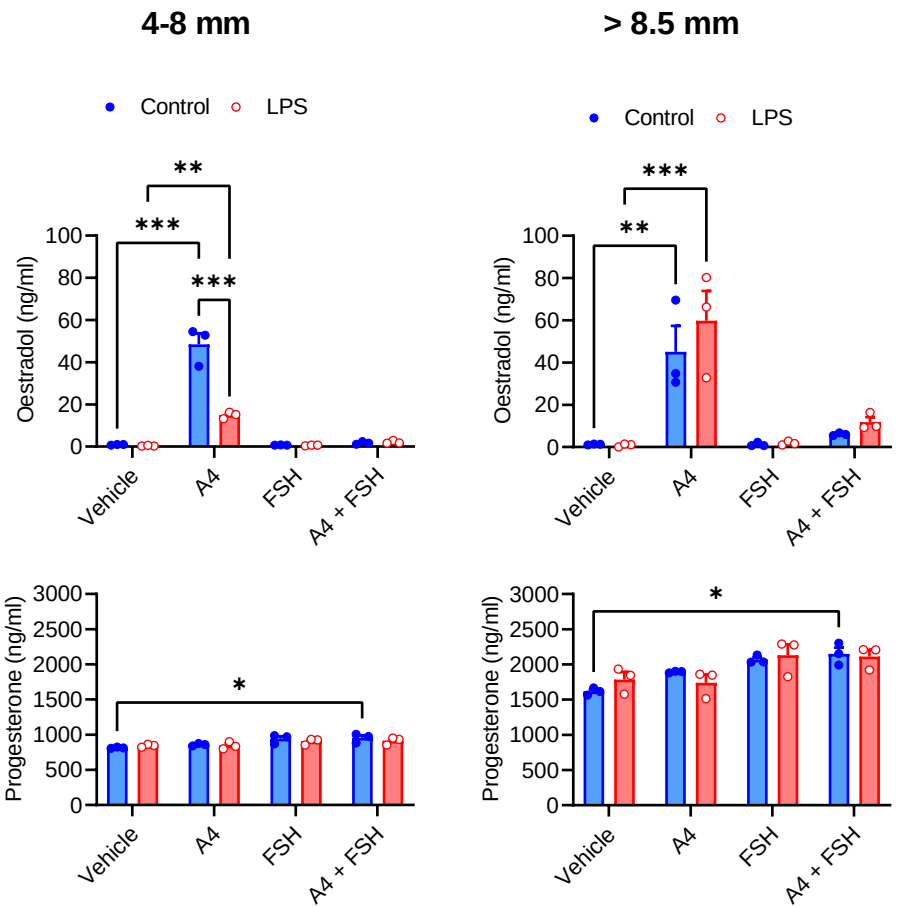
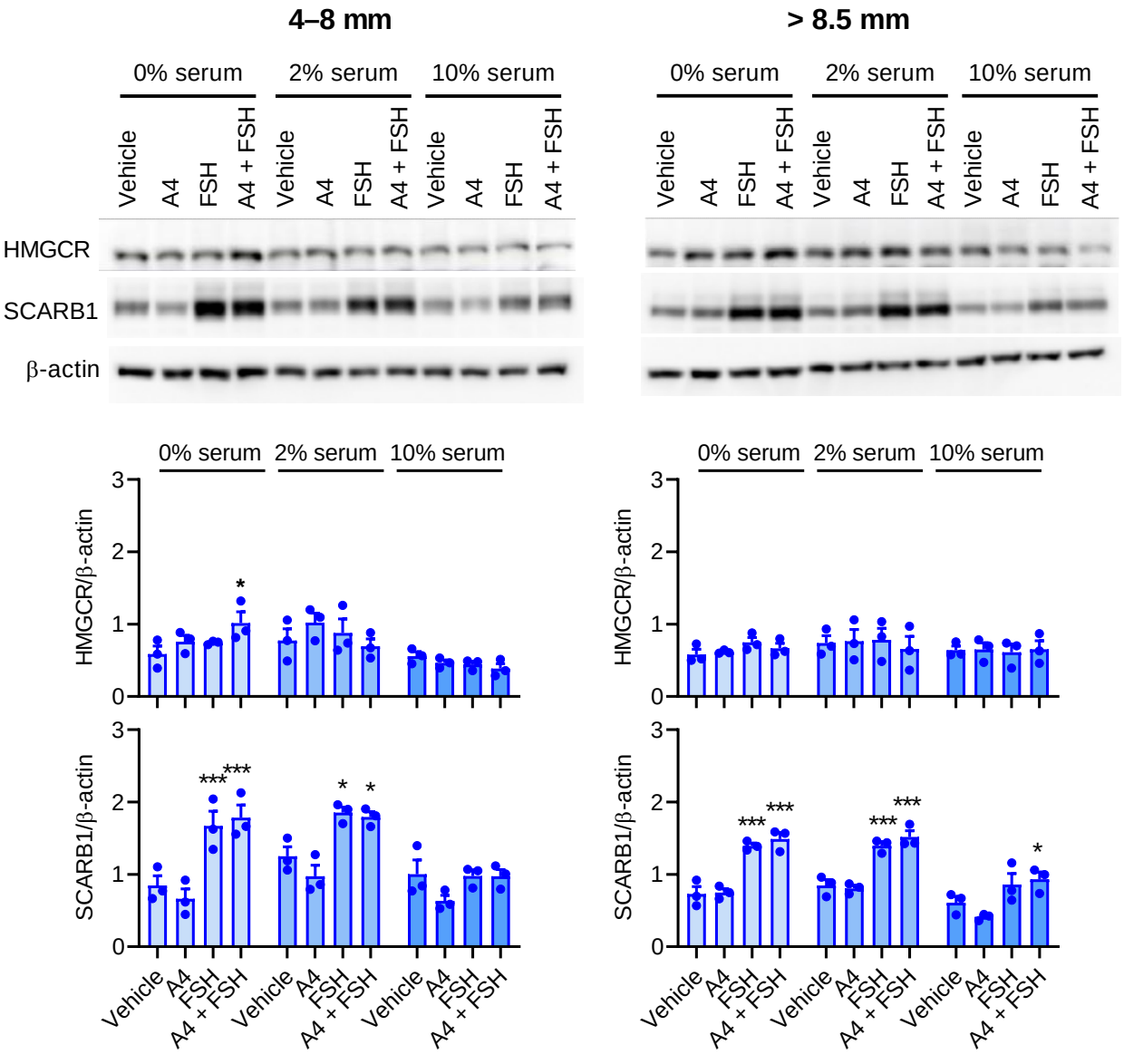

4-8 mm
> 8.5 mm
Control
LPS
Oestradol (ng/ml)
Progesterone (ng/ml)
Vehicle
A4
FSH
A4 + FSH
*
**
***

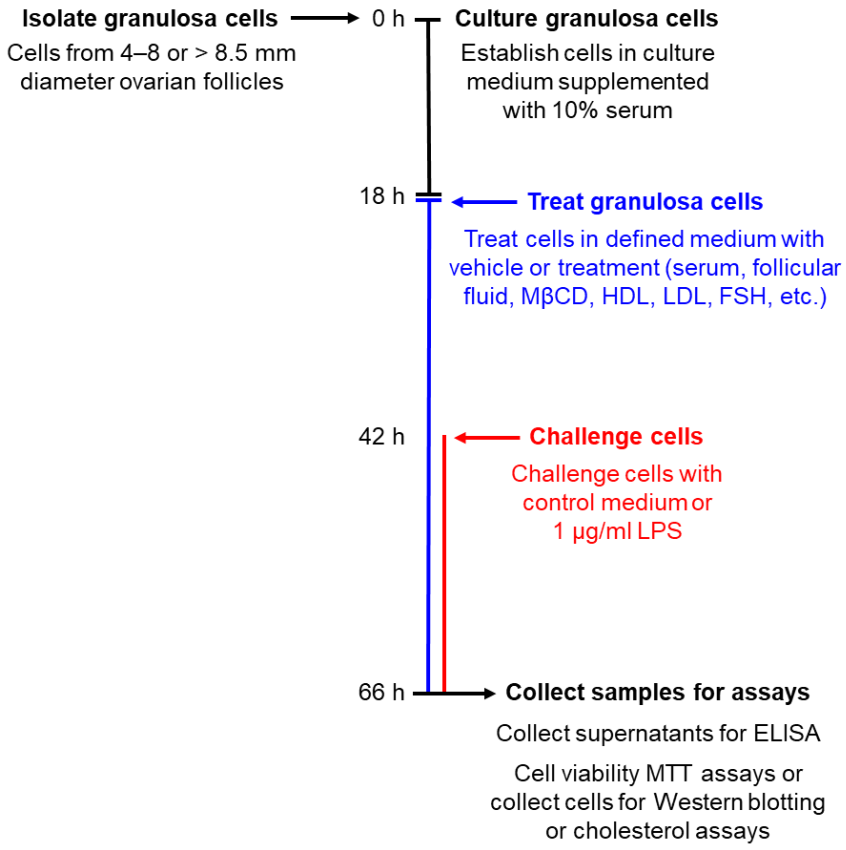
Figure 9


4–8 mm
> 8.5 mm
0% serum
2% serum
10% serum
Vehicle
A4
FSH
A4 + FSH
HMGCR
SCARB1
β-actin
HMGCR/β-actin
SCARB1/β-actin

Supplementary Data

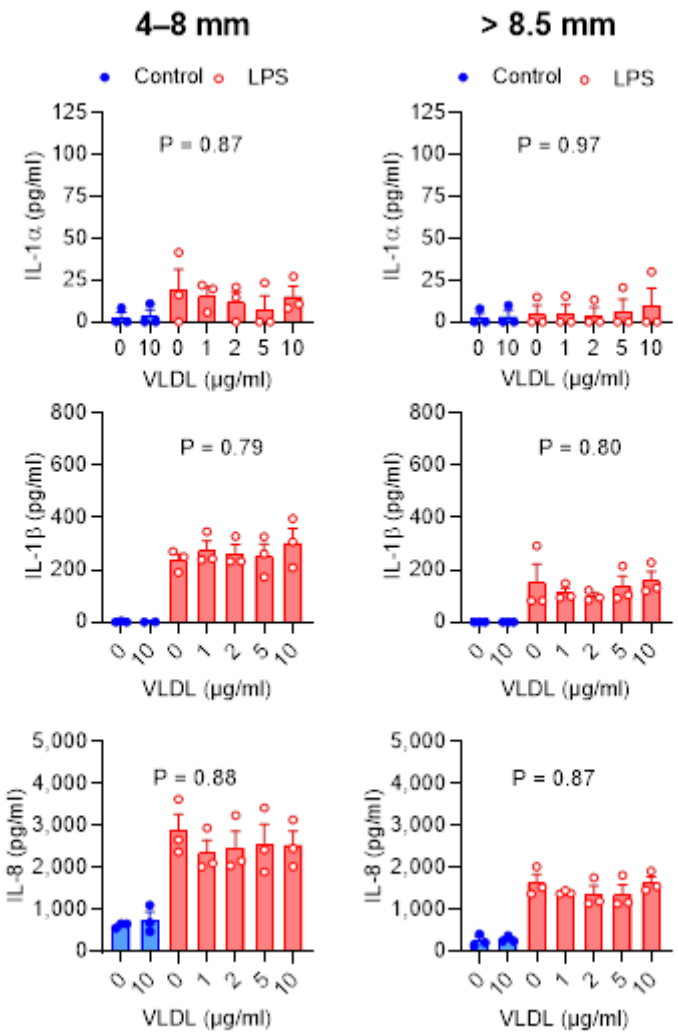
Cholesterol supports bovine granulosa cell inflammatory responses to lipopolysaccharide

AD Horlock, TJR Ormsby, MJD Clift, JEP Santos, JJ Bromfield and IM Sheldon



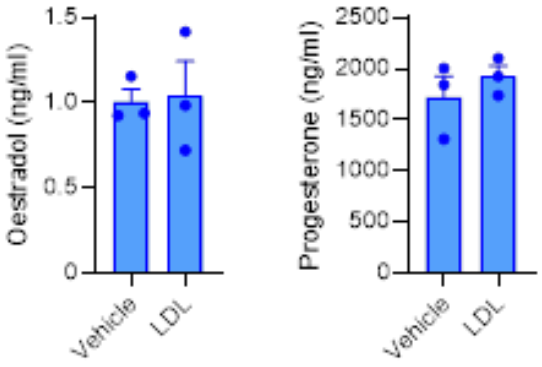
Supplementary Figure 1. Schematic of experimental protocol

Granulosa cells isolated from 4–8 mm or > 8.5 mm diameter ovarian follicles were grown in granulosa cell culture medium supplemented with 10% fetal bovine serum in 24-well plates for 18 h to achieve 80% confluence. The medium was discarded, and the cells were then treated in a granulosa cell culture medium (with or without serum) for 24 h with the treatments specified in the *Materials and Methods* section. The cells were finally challenged with control medium or medium containing 1 µg/ml ultrapure lipopolysaccharide (LPS) for a further 24 h in the continuing presence of the treatments. Samples were collected at the end of each experiment, including culture supernatants for ELISA and cells for protein or cholesterol analysis, or cell viability was evaluated by MTT assay. Each experiment was performed with 3 to 6 independent cultures of granulosa cells.



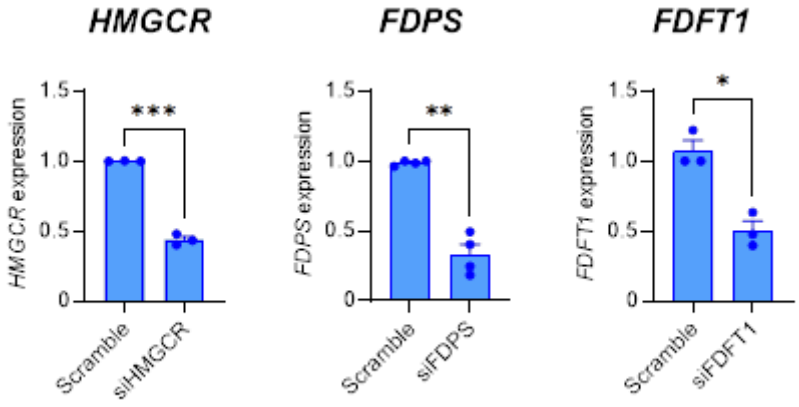
Supplementary Figure 2. VLDL cholesterol does not alter granulosa cell inflammatory responses to LPS

Granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles were cultured for 24 h with the indicated concentrations of VLDL cholesterol and then challenged for 24 h with control medium (●) or medium containing 1 µg/ml LPS (○), in the continued presence of the treatment. Cell supernatant IL-1α, IL-1β or IL-8 concentrations were measured by ELISA. Data are presented as mean + SEM from 3 independent experiments; statistical significance was determined using ANOVA, and P values reported for the effect of VLDL cholesterol on responses to LPS.



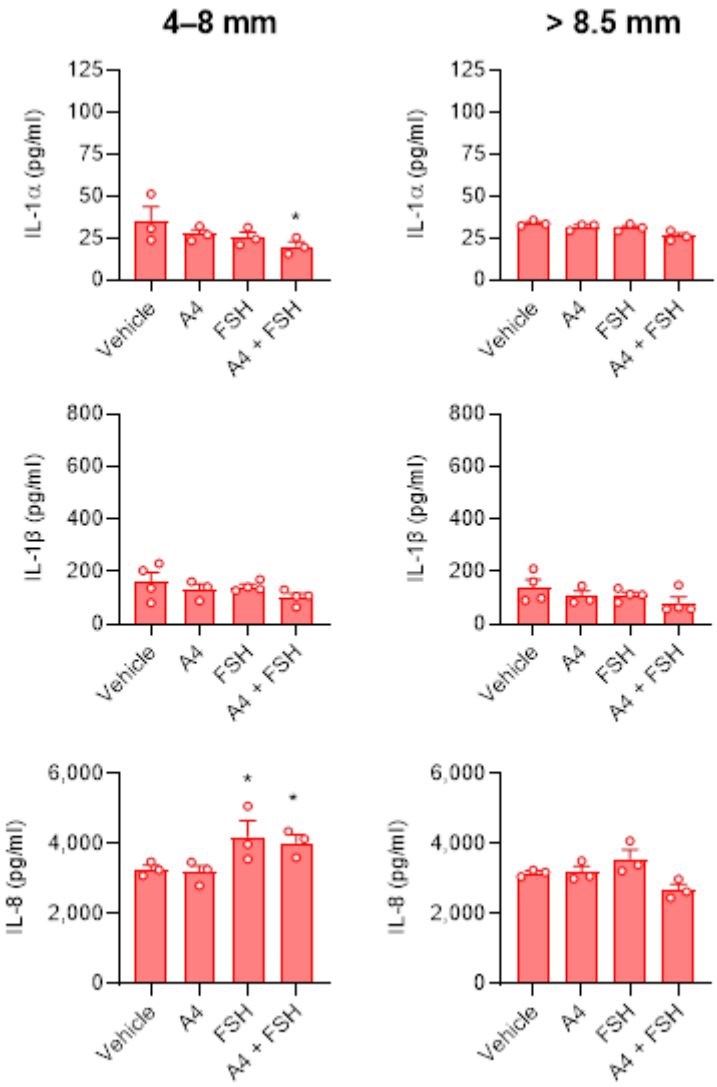
Supplementary Figure 3. LDL cholesterol does not alter oestradiol or progesterone secretion from granulosa cells

Granulosa cells from > 8.5 mm diameter ovarian follicles were treated for 24 h in serum-free culture medium containing either vehicle or 50 µg/ml LDL cholesterol. Cell supernatant oestradiol and progesterone concentrations were measured by ELISA. Data are presented as mean + SEM from 3 independent experiments.



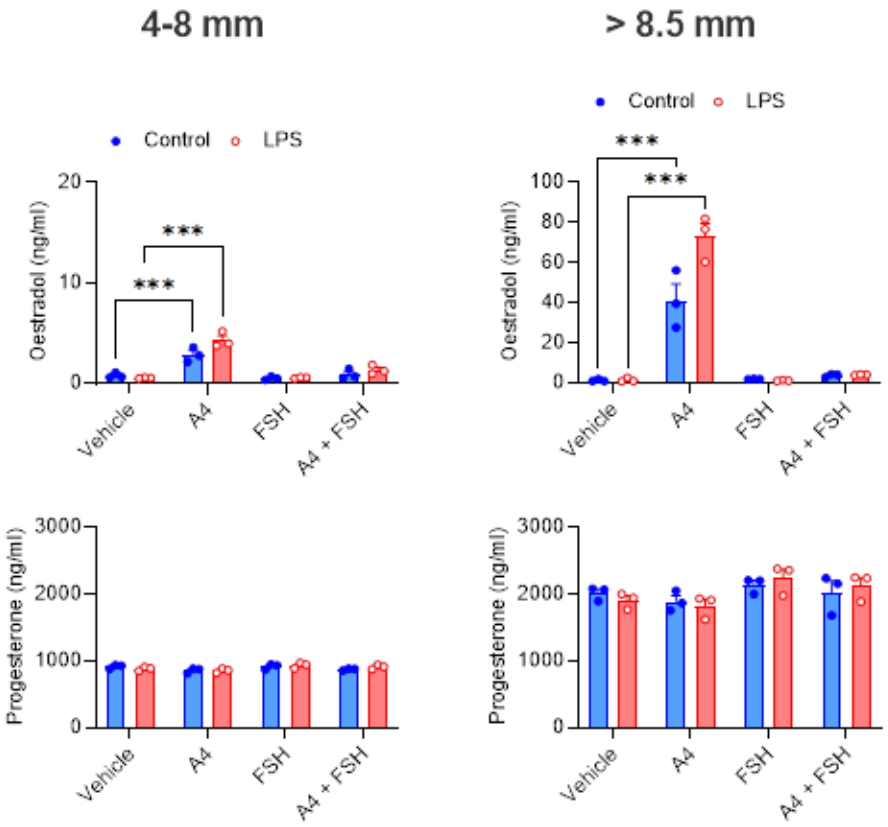
Supplementary Figure 4. Quantification of siRNA knockdown of *HMGCR*, *FDPS* and *FDFT1* in granulosa cells

Pooled populations of granulosa cells from 4–8 mm diameter and > 8.5 mm diameter ovarian follicles were transfected for 48 h with scramble siRNA or with siRNA targeting *HMGCR*, *FDPS* or *FDFT1*. The mRNA expression of each gene was measured by qPCR, and normalised to two reference genes (*ACTB* and *RLP19*). Data are presented as mean + SEM, from at least 3 independent experiments; statistical significance was determined using t-tests; values differ from scramble, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



Supplementary Figure 5. FSH and inflammatory responses to LPS with serum

Granulosa cells from 4–8 mm or > 8.5 mm diameter ovarian follicles were cultured in granulosa cell culture medium supplemented with 10% fetal bovine serum and treated for 24 h with vehicle, 10^{-7} M androstenedione (A4), 2.5 µg/ml FSH, or androstenedione and FSH, and then challenged for 24 h with 1 µg/ml LPS in the continued presence of the treatments. Cell supernatant IL-1α, IL-1β or IL-8 concentrations were measured by ELISA. Data are presented as mean + SEM from 3 independent experiments; statistical significance was determined using ANOVA and Dunnett's *post hoc* test, values differ from vehicle * $P < 0.05$.



Supplemental Figure 6. Androstenedione increases oestradiol secretion from granulosa cells cultured with serum

Granulosa cells isolated from 4–8 mm or > 8.5 mm diameter ovarian follicles were treated in granulosa cell culture medium supplemented with 10% fetal bovine serum for 24 h with vehicle, 10^{-7} M androstenedione (A4), 2.5 µg/ml FSH, or androstenedione and FSH, and then challenged for 24 h with control medium (●) or 1 µg/ml LPS (○) in the continued presence of the treatments. Cell supernatant oestradiol and progesterone concentrations were measured by ELISA. Data are presented as mean + SEM from 3 independent experiments. Statistical significance was determined using two-way ANOVA with Bonferroni's *post hoc* test; *** P < 0.001.