Ovarian steroids do not affect bovine endometrial cytokine or chemokine responses to *Escherichia coli* or LPS *in vitro*

João P E Saut^{1,2}, Gareth D Healey¹, Alan M Borges^{1,3} and I Martin Sheldon¹

¹College of Medicine, Institute of Life Science, Swansea University, Swansea, UK, ²Faculty of Veterinary Medicine, Federal University of Uberlândia, Campus Umuarama, Bloco 2T, Av. Pará, 1720, Bairro Umuarama, Uberlândia, Minas Gerais 38400-902, Brazil and ³Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Correspondence should be addressed to J P E Saut; Email: jpsaut@famev.ufu.br

Abstract

The risk of bacterial infection of the endometrium causing uterine disease in cattle is increased in the progesterone-dominated luteal phase of the ovarian cycle, while oestrogens or oestrus are therapeutic or protective against disease. The first line of defence against bacteria, such as *Escherichia coli* that cause inflammation of the endometrium, is the innate immune system, which recognises bacterial lipopolysaccharide (LPS). This study tested the hypothesis that cyclic variation in ovarian hormone concentrations alters innate immune responses within the bovine endometrium. *Ex vivo* organ cultures of endometrium, and *in vitro* cultures of endometrial epithelial and stromal cells, and peripheral blood mononuclear cells (PBMCs), all mounted inflammatory responses to *E. coli* or LPS, with secretion of inflammatory mediators interleukin 1 β (IL1 β), IL6 and IL8, and increased expression of mRNA encoding *IL1B*, *IL6*, *CXCL8* (*IL8*) and *CCL5*. However, these inflammatory responses, typical of innate immunity, were not affected by the stage of ovarian cycle in which the endometrium was collected for organ culture, or by exogenous oestradiol or progesterone. Although a dexamethasone-positive control reduced inflammation stimulated by *E. coli* or LPS, treatment with oestradiol or progesterone, or inhibitors of oestradiol or progesterone nuclear receptors, did not affect endometrial cell or PBMC secretion of IL1 β , IL6 or IL8, or *IL1B*, *IL6*, *CXCL8* and *CCL5* gene expression. In conclusion, the stage of the oestrus cycle or ovarian steroids did not modulate the innate immune response in the bovine endometrium *in vitro*.

Reproduction (2014) 148 593–606

Introduction

Microbial infections of the uterus are a common cause of infertility, abortion, pre-term labour and clinical disease of humans and animals (Wira *et al.* 2005, Turner *et al.* 2012). In dairy cows, *post partum* infection rates reach >90% after parturition, with clinical disease evident in nearly half of these cows. Disease of the uterus may persist for several weeks, is refractory to current treatments and leads to infertility (Sheldon *et al.* 2009). As a result, uterine disease of dairy cows is of economic importance, costing the EU dairy industry €1.4 billion/year (Sheldon *et al.* 2009). *Escherichia coli* are an important cause of pathology in the endometrium (Sheldon *et al.* 2010), with infection often preceding infection with *Trueperella pyogenes* and anaerobic bacteria (Sheldon *et al.* 2002). Furthermore, infection with *E. coli* is associated with negative effects on the ovary, hypothalamic–pituitary axis, and animal health and welfare (Williams *et al.* 2007). The endometrium forms an essential barrier to infection of the uterus.

Cytokines and chemokines orchestrate the recruitment and activation of immune cells to combat invading pathogens (Wira *et al.* 2005). Responses to microbial infection depend on pattern recognition receptors, such as the Toll-like receptors (TLRs), which are expressed by cells of the endometrium (Herath *et al.* 2006, 2009a, Sheldon & Bromfield 2011). In particular, endometrial epithelial and stromal cell responses to *E. coli* infection are mediated by TLR4, which binds lipopolysaccharide (LPS) leading to secretion of the chemokine interleukin 8 (IL8) and the cytokine IL6 (Cronin *et al.* 2012).

The endometrium undergoes physiological changes under the control of the ovarian steroids oestradiol and progesterone to create an environment suitable for pregnancy (Lewis 2003, Wira *et al.* 2005). These steroids also have an impact on endometrial disease. During the follicular phase of the oestrus cycle, when oestradiol concentrations are high, the endometrium is more resistant to infection, while the progesterone-dominated luteal phase of the oestrus cycle is associated with a

predisposition to development of disease (Rowson *et al.* 1953, Lewis 2003, 2004). Despite the clear effect of ovarian cycle on uterine disease progression, mechanistic data for the immune polarising effects of oestradiol and progesterone are less apparent. However, differences in uterine cellular profiles have been noted. Cells harvested from the uterine lumen around the time of ovulation secrete higher concentrations of cytokines and chemokines compared with cells harvested during the luteal phase of the oestrus cycle (Fischer *et al.* 2010). In some studies, ovarian steroids were associated with changes in neutrophil function (Roth *et al.* 1983), whereas in other studies, there were no consistent differences in peripheral leukocyte populations or their function (Subandrio & Noakes 1997, Subandrio *et al.* 2000, Winters *et al.* 2003). The inconsistency of leukocyte population differences and neutrophil functional changes suggest that steroid control of uterine disease progression may be the product of altered endometrial cell responses. Exogenous oestradiol and progesterone alter prostaglandin secretion *in vivo* in cows, sheep and pigs (Del Vecchio *et al.* 1992, Seals *et al.* 2002, Wulster-Radcliffe *et al.* 2003). *In vitro*, exogenous ovarian steroids reduce the secretion of prostaglandins by bovine epithelial and stromal cells stimulated with LPS (Herath *et al.* 2006). Therefore, we aimed to test whether the stage of the oestrus cycle or exogenous ovarian steroids might affect the innate immune response in the bovine endometrium using *ex vivo* studies to avoid confounding effects of humoral factors and adaptive immunity *in vivo*.

This study tested the hypothesis that cyclical variation in ovarian hormone concentrations alters cytokine and chemokine responses in bovine endometrial *ex vivo* organ cultures (EVOCs) and purified cell populations challenged with LPS or *E. coli*. Two main approaches were used. First, inflammatory responses to *E. coli* or LPS were examined in tissues collected from animals at different stages of the oestrus cycle. Secondly, tissues and cells were treated with exogenous oestradiol and progesterone or with inhibitors of the oestradiol or progesterone receptors (PRs). Comparisons were made to the glucocorticoid dexamethasone, which is an established modulator of innate immune responses (Kern *et al.* 1988, Waage & Bakke 1988).

Materials and methods

Organ and cell culture

Uteri with no gross evidence of genital disease or microbial infection were collected over a 10-month period from postpubertal mixed-breed beef heifers or dairy cows within 15 min of slaughter at a commercial slaughterhouse, as part of the routine operation of the slaughterhouse. Cattle up to 120 days *post partum* were not used to avoid confounding experiments due to the presence of ubiquitous bacterial

contamination and disruption of the epithelium, which is typical of the puerperal endometrium (Herath *et al.* 2009b, Wathes *et al.* 2009). The beef heifers ($n=174$) were 20–26 months old, reared on extensive grassland and had never been pregnant or inseminated. Dairy cows that were pregnant, as determined when the uterine horns were opened (see below), were excluded from the study. The stage of reproductive cycle was determined by examination of ovarian morphology and vasculature, as described previously in detail (Ireland *et al.* 1979) and by the measurement of hormones in peripheral blood. In accordance with these criteria, stage I is defined as the period from days 1 to 4 of the oestrus cycle, stage II as the period from days 5 to 10, stage III as the period from days 11 to 17 and stage IV as the period from days 18 to 20. Only animals that had gross evidence of ovarian cyclic activity were included. To further evaluate the stage of ovarian cycle, blood samples were collected from the animal carcass at the time of uteri collection, allowed to clot at room temperature and then centrifuged at 2000 *g* for 15 min to separate the serum, which was then divided in aliquots of 1.5 ml in Eppendorf tubes and frozen at -80°C until used for progesterone analysis (see below). Within this study, animals from stages IV and I of the oestrus cycle were grouped together, as this represents the follicular phase when serum progesterone concentration is <1 ng/ml.

The uteri were kept on ice for ~ 1 h until further processing at the laboratory. Endometrial tissue for EVOC was collected from the contralateral horn, unless otherwise stated, and the intercaruncular areas of the endometrium, except for comparison of responses between intercaruncular and caruncular tissue, using sterile 8 mm-diameter biopsy punches (Stiefel Laboratories Ltd, High Wycombe, UK), as described previously (Borges *et al.* 2012). Tissues were cultured in 24-well plates (TPP, Trasadingen, Switzerland) containing 2 ml complete medium/well, and comprised the following: phenol red-free RPMI 1640 medium (Sigma–Aldrich) containing 10% heat inactivated, double charcoal-stripped, foetal bovine serum (Biosera, East Sussex, UK). The EVOC treatments (see below) were initiated within 4 h of slaughter and maintained in a humidified, 5% CO_2 in air atmosphere incubator at 37°C , with supernatants collected after 6, 24 or 48 h.

Endometrial cells were isolated as described previously (Cronin *et al.* 2012, Turner *et al.* 2014). The epithelial and stromal cells were cultured in a complete medium and plated at a density of 1×10^5 cells/ml in 24-well plates (TPP). The purity of epithelial and stromal cell populations was confirmed by cell morphology and flow cytometric analysis of cytokeratin and vimentin expression respectively (Fortier *et al.* 1988, Turner *et al.* 2014).

The isolation and culture of peripheral blood mononuclear cells (PBMCs) were performed as described previously (Herath *et al.* 2007, Amos *et al.* 2014). Cells were seeded into 24-well plates at a density of 1×10^6 cells/well in 1 ml of complete medium and the medium was changed every 2 days until cells exhibited the characteristic macrophage morphology (Steinman & Cohn 1973). The cell population phenotype, which was CD14+, CD45+ and MHC class II+, was confirmed by flow cytometry as described previously (Herath *et al.* 2007, Price *et al.* 2013).

Experimental design

Treatments

Cultures of *E. coli* (isolate MS499) obtained from an animal with persistent uterine disease, and identified as an endometrial pathogenic *E. coli* (Sheldon *et al.* 2010, Goldstone *et al.* 2014), were grown overnight in Luria-Bertani medium (Sigma–Aldrich). Bacteria were re-suspended to 1×10^8 colony forming units (CFU)/ml in sterile PBS (Life Technologies Ltd), followed by centrifugation at 6000 *g* for 10 min at 4 °C. After washing, bacteria were diluted to 1×10^3 CFU/ml in a complete medium ready for experimental use. Ultrapure LPS from *E. coli* 0111:B4 was obtained from Invivogen (Toulouse, France). Ovarian and glucocorticoid steroids (E_2 , E2758; progesterone, P8783 and dexamethasone, D4902) and the steroid receptor antagonists (MPP dihydrochloride hydrate, M7068 and mifepristone, M8046) were obtained from Sigma–Aldrich Ltd. Ovarian and glucocorticoid steroids were prepared by dissolving 1 mg of the steroid in 1 ml absolute ethanol. Stock solutions were prepared at a concentration of 20 µg/ml in complete medium. Final concentrations of E_2 , progesterone and dexamethasone were prepared by further dilutions in a complete medium. The final concentration of ethanol within tissue or cell cultures was equal to or less than one part in 200 000. MPP dihydrochloride hydrate was prepared by dissolving 20 mg in 1 ml dimethyl sulfoxide. A stock solution was prepared at a concentration of 1 mg/ml (1.85 mM) in complete medium. The final concentration of MPP dihydrochloride hydrate (100 nM) was prepared by further dilution in complete medium. The final concentration of dimethyl sulfoxide within tissue of cell cultures was one part in 370 000. Mifepristone was prepared by dissolving 10 mg in 1 ml absolute ethanol. A stock solution was prepared at a concentration of 1 mg/ml (2.32 mM) in a complete medium. The final concentration of mifepristone (100 nM) was prepared by further dilution in complete medium. The final concentration of ethanol within tissue of cell cultures was one part in 232 000. All treatments were carried out in complete medium that did not contain antibiotics to ensure that bacteria were alive and replicating.

Validation of innate immune responses of EVOCs

To compare endometrial innate immune responses between beef heifers and dairy cows, EVOCs were prepared from beef heifer ($n=9$) and dairy ($n=7$) cow uteri that were within the early luteal phase of the oestrus cycle. Comparison of EVOCs using endometrial tissue from intercaruncular ($n=4$) and caruncular ($n=4$) zones of the endometrium was performed using early-luteal-phase beef heifer uteri. Endometrial EVOCs were treated with the control medium or a medium containing 1 µg/ml LPS or 1×10^3 CFU/ml *E. coli* for 24 h. Comparison of EVOCs using endometrial tissue from the horn ipsilateral ($n=13$) and contralateral ($n=29$) to the active corpus luteum (CL) structure was performed using early-luteal-phase beef heifer uteri. Endometrial EVOCs were treated with the control medium or a medium containing 1 µg/ml LPS for 24 h. After treatment, supernatants were collected and stored at –20 °C for analysis of IL1β, IL6 and IL8 by ELISA. The EVOc tissues were weighed and stored in 0.5 ml TRI Reagent at –20 °C until

RNA extraction and analysis of *IL1B*, *IL6* and *CXCL8* (*IL8*) mRNA expression by quantitative PCR (qPCR).

Uterine innate immune responses and stage of the oestrus cycle

To evaluate the effect of the stage of the oestrus cycle, tissues from 55 beef heifers were divided into three groups by examination of ovarian morphology (Ireland *et al.* 1979) and retrospective serum progesterone analysis (see below): follicular phase was defined by ovarian stage (Ireland *et al.* 1979, stages IV and I) with a serum progesterone concentration of <1 ng/ml ($n=6$), early luteal phase (Ireland *et al.* 1979, stage II) with a serum progesterone concentration of 1–2 ng/ml ($n=10$) and mid-luteal phase (Ireland *et al.* 1979, stage III) with a serum progesterone concentration of >2 ng/ml ($n=39$). Endometrial EVOCs from each group were treated with the control medium or a medium containing 1 µg/ml LPS or 1×10^3 CFU/ml *E. coli* for 6 or 24 h. After treatment, the supernatants were collected and stored at –20 °C for analysis of IL1β, IL6, IL8 and PGE₂ by ELISA. The EVOc tissues were weighed and stored in 0.5 ml TRI Reagent at –20 °C until RNA extraction and analysis of *IL1B*, *IL6*, *CXCL8* and *CCL5* mRNA expression by qPCR.

Steroids and innate immune responses within ex vivo organ cultures

A total of 76 beef heifers were used to evaluate the effect of ovarian and glucocorticoid steroids on innate immune responses within the bovine endometrium. Endometrial EVOCs were divided into two groups according to a retrospective analysis of serum progesterone concentration: group 1 with a serum progesterone concentration of <2 ng/ml ($n=20$) and group 2 with a serum progesterone concentration of >2 ng/ml ($n=56$). Endometrial EVOCs were pre-treated for 24 h with the control medium or a medium containing 3 pg/ml oestradiol, 5 ng/ml progesterone or 5 ng/ml dexamethasone. The concentrations of oestradiol and progesterone reflect serum concentrations approximately at the time of ovulation and during the luteal phase of the oestrus cycle respectively (Sheldon *et al.* 2002, Jimenez-Krassel *et al.* 2009, Scully *et al.* 2014). The concentration of dexamethasone used was based upon the recommended potency range from the manufacturer (4–500 ng/ml) and a previous publication, with the aim being to use a minimal effective dose (Kern *et al.* 1988). After 24 h, EVOCs were challenged with the control medium or a medium containing 1 µg/ml LPS or 1×10^3 CFU/ml *E. coli* for a further 24 h in the presence of the steroids. After challenge, supernatants were collected and stored at –20 °C for analysis of IL1β, IL6 and IL8 by ELISA. The EVOc tissues were weighed and stored in 0.5 ml TRI Reagent at –20 °C for analysis of *IL1B*, *IL6*, *CXCL8* and *CCL5*. In addition, the expression of *ESR1* and *PGR* mRNA was determined by qPCR to ensure that the tissue remained responsive to exogenous steroids for the 48 h treatment period.

Steroids and cellular innate immune responses

To evaluate the impact of ovarian and glucocorticoid steroids on *in vitro* cellular innate immune responses, endometrial

epithelial and stromal cells and PBMCs, collected from four beef heifers, were pre-treated for 24 h with the control medium or a medium containing 3 pg/ml oestradiol, 5 ng/ml progesterone or 5 ng/ml dexamethasone. After 24 h, cells were challenged with the control medium or a medium containing 100 ng/ml LPS for a further 24 h in the presence of the steroids. After challenge, supernatants were collected and stored at -20°C for analyses of IL6 and IL8 by ELISA. Additionally, endometrial stromal cells from three beef heifers were pre-treated for 24 h with the control medium or a medium containing 1–30 pg/ml oestradiol, 1–30 ng/ml progesterone or 5 ng/ml dexamethasone. After 24 h, cells were challenged with the control medium or a medium containing 100 ng/ml LPS for a further 24 h in the presence of the steroids. After challenge, supernatants were collected and stored at -20°C for analysis of IL6 and IL8 by ELISA. Cell survival was assessed by the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, as described previously (Mosmann 1983). Briefly, supernatants were removed and replaced with fresh complete medium containing 0.5 mg/ml MTT before being incubated with cells at 37°C in a humidified atmosphere of 5% CO_2 for 1 h. The medium was then removed and cells were washed with sterile PBS before lysis with dimethyl sulfoxide and measurement of the optical density was performed at a wavelength of 570 nm using a microplate reader (POLARstar Omega; BMG Labtech, Offenburg, Germany). The correlation between MTT OD 570 measurements and the number of live cells was confirmed using Trypan blue exclusion and counting the number of live cells using a haemocytometer.

Steroid receptor antagonists and endometrial innate immune responses

To further explore the impact of steroids on immunity in the endometrium, their actions were inhibited using antagonists for their nuclear receptors. Endometrial EVOCs from 23 beef heifers in the luteal phase of the oestrus cycle were pre-treated for 24 h with the control medium or a medium containing 5 ng/ml progesterone, 3 pg/ml oestradiol or 5 ng/ml dexamethasone. Pre-treatments were performed in the presence or absence of the oestrogen receptor alpha ($\text{ER}\alpha$) antagonist MPP dihydrochloride hydrate (MPP, 100 nM) (Sun *et al.* 2002) or the PR/glucocorticoid receptor (GR) antagonist mifepristone (100 nM) (Skinner *et al.* 1999, Siemieniuch *et al.* 2010). After 24 h, the EVOCs were challenged with the control medium or a medium containing 1 $\mu\text{g/ml}$ LPS or 1×10^3 CFU/ml *E. coli* for a further 24 h in the presence of the steroids and/or antagonists. After challenge, the supernatants were collected and stored at -20°C for analysis of IL1 β , IL6 and IL8 by ELISA, and EVOC tissues were weighed.

Enzyme immune assays

Concentrations of IL1 β , IL6 and IL8 in EVOC and cell culture supernatants were measured in duplicate by ELISA according to the manufacturer's instructions (Bovine IL1 β Screening Set (ESS0027; Thermo Fisher Scientific, Perbio Science UK Ltd, Cramlington, UK); Bovine IL6 Screening Set (ESS0029; Thermo Fisher Scientific)

and Human CXCL8/IL8 DuoSet (DY208; R&D Systems Europe Ltd, Abingdon, UK)). The human CXCL8/IL8 DuoSet has previously been validated for the measurement of bovine IL8 (Rinaldi *et al.* 2008). To take into account the differences between the weights of EVOC tissues, concentrations are expressed in picograms per milligram of tissue. Serum progesterone concentrations were determined by a Progesterone Enzyme Immunoassay (Ridgeway Research Ltd, St Briavels, UK), according to the manufacturer's instructions. The inter- and intra-assay coefficient of variation values were all <12 and 7% respectively; the limits of detection were 12.5 pg/ml for IL1 β , 75.0 pg/ml for IL6, 5.7 pg/ml for IL8 and 0.1 ng/ml for progesterone.

Gene expression analysis

Gene expression analysis was performed according to the MIQE guidelines (Bustin *et al.* 2009). Total RNA was isolated from EVOC tissues by homogenising the tissue in 2 ml tubes containing 0.5 ml TRI Reagent (Sigma-Aldrich) and lysing matrix D (MP Biomedicals, Cambridge, UK) at a rate of 6.0 m/s for 2 min. After homogenisation, tubes were centrifuged at 12 000 *g* for 10 min, the supernatants were transferred to fresh 2 ml Eppendorf tubes, and RNA extraction from TRI Reagent was then performed according to the manufacturer's instructions. RT of 1 μg mRNA was performed in a 20 μl reaction volume using the QuantiTect RT Kit (Qiagen), according to the manufacturer's instructions.

QPCR for *IL1B*, *CXCL8*, *CCL5*, *ESR1*, *GAPDH* and *ACTB* was performed by multiplex probe-based PCR and comprised two panels of primer/probe combinations (panel 1: *IL1B*, *CXCL8* and *GAPDH* and panel 2: *CCL5*, *ESR1* and *ACTB*), which simultaneously measured cDNA for each target gene. PCR primers and probes were designed using the Eurofins MWG Operon qPCR primer/probe design software (https://ecom.mwgdna.com/services/webgist/dual_probe_design?usca_p=t) and validated by BLAST analysis against the *Bos taurus* (taxid:9913) Refseq mRNA database. Primers/probes were obtained from Eurofins MWG Operon (Ebersberg, Germany). Multiplex qPCR was performed in 10 μl reaction volumes comprising 1 $\times$ QuantiFast Multiplex PCR Master Mix (Qiagen) with primers and probes added in nuclease-free water to a final concentration of 0.4 and 0.2 μM , respectively, and 2 μl of cDNA. Thermal cycling parameters were as follows: one cycle of 95°C for 5 min followed by 50 cycles of 95°C for 15 s and 60°C for 30 s.

QPCR for *IL6* and *PGR* was performed by SYBR Green-based PCR because existing primers/methods for these genes were already present in the laboratory. PCR primers were designed using the Eurofins MWG Operon qPCR primer/probe design software (https://ecom.mwgdna.com/services/webgist/dual_probe_design?usca_p=t) and validated by BLAST analysis against the *Bos taurus* (taxid:9913) Refseq mRNA database. *PGR* and *IL6* primers were obtained from Eurofins MWG Operon and Sigma-Aldrich respectively. SYBR Green-based PCR was performed in a 25 μl reaction volume comprising 1 $\times$ QuantiFast SYBR Green PCR Master Mix (Qiagen) with primers added in nuclease-free water to a final concentration of 0.4 μM and 2 μl of cDNA. Thermal cycling parameters were as follows: one cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 60°C for 60 s.

Table 1 Quantitative PCR primers and probes used for gene expression analysis.

Genes	Primers/probes (5'–3')
<i>IL1B</i>	Forward: TCCTATTCTCTCCAGCCA Reverse: AGCCTCAAATAACAGCTCATTC Probe: FAM-ATGGCAACCGTACCTGAACCCATCAA-BHQ1
<i>CXCL8</i>	Forward: CACATTCCACACCTTTCCAC Reverse: CCTTCTGCACCCACTTTTC Probe: Hex-GGAAACGAGTCTGCCTAAACCCCAA-BHQ1
<i>GAPDH</i>	Forward: ATTCCACCCACGGCAAGTTC Reverse: TCCATCGTCCACCGCAAATGCTTCT Probe: Cy5-GCAGAGAACGGGAAGCTCGTCATCAATGGAA-BBQ650
<i>CCL5</i>	Forward: CTTTGCCTATATCTCCCGCC Reverse: TCTCGCACCCACTTCTTCTC Probe: FAM-CAGCAGTTGTCTTTATCACCAGGAAGAAGCGCCA-BHQ1
<i>ACTB</i>	Forward: AAGAAAAAGGGTGTAAACGCAG Reverse: TCCATCGTCCACCGCAAATGCTTCT Probe: LC705-ATTCCACCCACGGCAAGTTC-BBQ650
<i>ESR1</i>	Forward: ACTCTCTCTATCTCTCTC Reverse: CACCACGTTCTTGCACTTC Probe: Hex-GGCACATGAGCAACAAAGGCATGGA-BHQ1
<i>IL6</i>	Forward: ATGACTTCTGCTTTCCCTACCC Reverse: GCTGCTTTCACTCATCATTC
<i>PGR</i>	Forward: CGTGGAGGGGGCGTATCCG Reverse: CGGGGCCAAAGAGGCCACCA

All primers and probes used are given in detail in Table 1. The expression of each gene was normalised against the geometric mean of the reference genes *GAPDH* and *ACTB*, which were invariant across treatment groups (Vandesompele *et al.* 2002), and the relative quantification method was employed to quantify target gene mRNA within samples (Nolan *et al.* 2006). To generate standard curves, total RNA extracted from EVOc tissues that had been treated with 1 µg/ml LPS for 24 h was reverse transcribed to cDNA, as described. Tenfold serial dilutions of this reference cDNA were prepared (neat to 1×10^{-5}) in nuclease-free water (Qiagen). For each sample, target and reference gene mRNA abundance was determined from the appropriate standard curve (quantification cycle (C_q)). Changes in mRNA abundance between samples were then determined from the ratio of the target gene C_q to the reference gene C_q .

Statistical analysis

Statistical analyses were performed using SAS version 8.0 with the animal as the experimental unit. Initially, the data were tested for homogeneity, followed by analysis using the general linear model (GLM) multiplex ANOVA using Dunnett's pairwise multiple comparison *t*-test for individual group comparisons. Gene data are expressed as dot plots, protein data are expressed as histograms and data are expressed as mean with S.E.M. $P < 0.05$ was considered statistically significant.

Results

Endometrial EVOcs from beef heifers and dairy cows respond similarly to LPS and *E. coli*

To validate the utility of the EVOc system, selected cytokine and chemokine responses were compared between EVOcs from beef heifer and dairy cow endometria. Endometrial EVOcs from beef heifers and dairy cows accumulated more IL1β, IL6 and IL8 following challenge with LPS or *E. coli* compared with the control

medium (Fig. 1A, B and C, $P < 0.0001$). There was also increased *IL1B*, *IL6* and *CXCL8* mRNA expression in response to challenge with LPS or *E. coli* (Fig. 1D, E and F, $P < 0.0001$). However, there was no significant difference in the protein or mRNA responses to LPS or *E. coli* between EVOcs from beef heifers and dairy cows.

To further validate the use of EVOcs, inflammatory responses to *E. coli* and LPS were compared between caruncular and intercaruncular EVOcs collected from beef heifer endometrium. Endometrial EVOcs from both areas secreted more IL1β, IL6 and IL8 in response to challenge with LPS or *E. coli* compared with the control medium (Fig. 1G, H and I, $P < 0.05$). However, more IL1β, IL6 and IL8 were secreted from intercaruncular EVOcs compared with caruncular EVOcs following challenge with LPS ($P < 0.05$), and more IL1β and IL6 were secreted following challenge with *E. coli* ($P < 0.05$).

Finally, inflammatory responses to LPS were compared between endometrial EVOcs collected from the horn ipsilateral and contralateral to the active CL structure. Endometrial EVOcs from both horns secreted more IL6 in response to challenge with LPS compared with the control medium (Fig. 1K, $P < 0.001$), and there was a trend for increased IL1β and IL8. Importantly, however, there were no significant differences in responses to LPS between EVOcs from the ipsilateral or contralateral horns.

Endometrial innate immune responses and stage of the oestrus cycle

To investigate the role of the oestrus cycle in modulating innate immunity, responses to challenge with LPS or *E. coli* for 6 or 24 h were tested using EVOcs of intercaruncular endometrium collected from beef animals at different stages of the oestrus cycle. As expected, cows in the mid-luteal phase had more progesterone present in their serum (8.51 ± 0.75 ng/ml, $P < 0.001$) compared with cows in the follicular (0.08 ± 0.07 ng/ml) or early luteal (0.5 ± 0.21 ng/ml) phases.

Following a 6 h challenge with LPS or *E. coli*, EVOcs accumulated more IL6 ($P < 0.0001$), compared with the control medium, and after a 24 h challenge with LPS or *E. coli*, EVOcs accumulated more IL1β, IL6 and IL8 ($P < 0.0001$) (Fig. 2). Mid-luteal-phase cow EVOcs accumulated more IL6 ($P < 0.05$), but not IL1β or IL8, in response to a 6 h challenge with LPS compared with follicular- and early-luteal-phase cow EVOcs (Fig. 2A, B and C). There were no significant differences between the stages of the oestrus cycle following a 6 h challenge with *E. coli*. Mid-luteal-phase cow EVOcs accumulated less IL1β ($P < 0.05$) compared with early-luteal-phase cow EVOcs following a 24 h challenge with *E. coli*, but there was no difference in IL6 or IL8 secretion (Fig. 2D, E and F). There were no significant differences between stages of the oestrus cycle for EVOcs challenged with LPS for 24 h. To further explore the impact of the stage of the oestrus cycle on inflammatory responses in the

Figure 1 Ex vivo organ culture responses to LPS or *Escherichia coli*. Endometrial EVOCs were collected from the intercaruncular areas (contralateral horn) of early-luteal-phase beef heifer (open bars or open circle) and dairy cow (closed bars or closed square) endometria, and challenged with the control medium or a medium containing 1 μ g/ml LPS or 1×10^3 CFU/ml *E. coli* for 24 h. Supernatants were collected and analysed for IL1 β , IL6 and IL8 by ELISA (A, B and C: beef heifers, $n=9$ and dairy cows, $n=7$) and IL1B, IL6 and CXCL8 mRNA expression by qPCR (D, E and F: beef heifers, $n=4$ and dairy cows, $n=4$). Endometrial EVOCs collected from the caruncular (closed bars, $n=4$) and intercaruncular (open bars, $n=4$) areas of early-luteal-phase beef heifer endometrium (contralateral horn) were challenged with the control medium or a medium containing 1 μ g/ml LPS or 1×10^3 CFU/ml *E. coli* for 24 h. Supernatants were collected and analysed for IL1 β , IL6 and IL8 by ELISA (G, H and I). Endometrial EVOCs collected from the intercaruncular areas of the ipsilateral (open bars, $n=13$) and contralateral (closed bars, $n=13$) horns of early-luteal-phase beef heifer endometrium were challenged with the control medium or a medium containing 1 μ g/ml LPS for 24 h. Supernatants were collected and analysed for IL1 β , IL6 and IL8 by ELISA (J, K and L). Data are expressed as mean (s.e.m.), protein data as histograms, and qPCR data as dot plots with the horizontal bar representing the mean and vertical bars s.e.m. Data were analysed by GLM multivariate ANOVA and Dunnett's pairwise multiple comparison t -test and values differ between groups: $*P < 0.05$.

Figure 2 *Ex vivo* organ culture responses are not affected by the stage of the oestrus cycle. Endometrial EVOCs collected from the intercaruncular areas of beef heifer endometrium (contralateral horn) at different stages of the oestrus cycle were challenged with the control medium or a medium containing 1 µg/ml LPS or 1×10^3 CFU/ml *Escherichia coli* for 6 (A, B and C: follicular, open bars, $n=3$; early luteal, grey bars, $n=10$ and mid luteal, closed bars, $n=20$) or 24 h (D, E and F: follicular, open bars, $n=6$; early luteal, grey bars, $n=10$ and mid luteal, closed bars, $n=39$). Supernatants were collected and analysed for IL1β, IL6 and IL8 by ELISA. Data are expressed as mean (S.E.M.). Data were analysed by GLM multivariate ANOVA and Dunnett's pairwise multiple comparison *t*-test and values differ between groups: * $P<0.05$.

endometrium, the EVOc data were combined (follicular, $n=19$; early-luteal, $n=43$ and mid-luteal, $n=22$). Treatment with LPS or *E. coli* increased the accumulation of IL6 compared with the control (305.7 ± 13.1 , 243.4 ± 12.4 and 93.1 ± 13.9 pg/ml, respectively; ANOVA, $P<0.001$). However, there was no significant effect of the stage of cycle ($P=0.23$) or the interaction between treatment and stage ($P=0.88$).

Challenge of EVOCs with *E. coli* or LPS for 6 or 24 h also increased the expression of *IL1B*, *IL6*, *CXCL8* and *CCL5* (Fig. 3, $P<0.05$). However, there were no consistent differences in mRNA expression among the different stages of the oestrus cycle measured for any of the four genes examined at 6 or 24 h (Fig. 3).

Ovarian steroids and endometrial responses to LPS or *E. coli*

In the absence of an effect of the stage of the oestrus cycle on the inflammatory response, an alternative approach was examined to test whether ovarian steroids regulate endometrial innate immunity, by treating EVOCs with exogenous steroids before challenge with LPS or *E. coli*. The EVOCs were retrospectively divided into two groups, based on the serum progesterone concentration: <2 ng/ml (follicular) and >2 ng/ml (luteal). As ovarian hormones regulate the *ESR1* and *PGR* genes (Kimmins & MacLaren 2001), to confirm the responsiveness of the

EVOCs to steroid treatment, the impact of oestradiol, progesterone and the glucocorticoid dexamethasone on *ESR1* and *PGR* mRNA expression was measured. Within EVOCs of follicular-phase endometrial tissue, 48 h treatment with E_2 increased the expression of *ESR1* (Fig. 4A, $P<0.05$) and *PGR* (Fig. 4C, $P<0.05$) mRNA compared with the control, and progesterone significantly decreased the expression of *PGR* (Fig. 4C, $P<0.05$) mRNA, while dexamethasone had no effect (Fig. 4A and C). The same pattern of change in expression of *PGR* mRNA was measured in EVOCs obtained from luteal-phase cows (Fig. 4D, $P<0.05$), but none of the steroids had any significant effect on *ESR1*.

As mentioned previously, irrespective of steroid treatment, EVOCs responded to challenge with LPS by accumulating IL6 and IL8, and accumulated IL6, IL8 and IL1β in response to challenge with *E. coli* (Fig. 5, $P<0.05$). However, pre-treatment of EVOCs with either oestradiol or progesterone for 24 h had no significant effect on responses to subsequent challenges with LPS or *E. coli*, and there was no effect of the prior hormone concentration in the animals. However, dexamethasone reduced the accumulation of IL1β in EVOCs before and following challenge with LPS or *E. coli* (Fig. 5A and B, $P<0.05$). As mentioned previously, challenge of EVOCs with LPS or *E. coli* increased the expression of *IL1B*, *IL6*, *CXCL8* and *CCL5* mRNA compared with the control medium (Fig. 6, $P<0.001$). However, pre-treatment with

Figure 3 Gene expression within *ex vivo* organ cultures is not affected by the stage of the oestrus cycle. Endometrial EVOCs collected from the intercaruncular areas of beef heifer endometrium (contralateral horn) at different stages of the oestrus cycle were treated with the control medium or a medium containing 1 $\mu\text{g/ml}$ LPS or 1×10^3 CFU/ml *Escherichia coli* for 6 or 24 h. For 6 h treatment: follicular (closed circle), $n=3$, early luteal (open square), $n=4$ and mid-luteal (open triangle), $n=10$. For 24 h treatment: follicular (closed circle), $n=3$, early luteal (open square), $n=4$ and mid-luteal (open triangle), $n=9$. The EVOC tissues were collected and total RNA was extracted for analysis of *IL1B* (A and B), *IL6* (C and D), *CXCL8* (E and F) and *CCL5* (G and H) mRNA expression by qPCR. Data are expressed as dot plots with the horizontal bar representing the mean and vertical bars S.E.M. Data were analysed by GLM multivariate ANOVA and Dunnett's pairwise multiple comparison *t*-test.

oestradiol or progesterone had no effect on mRNA abundance in response to these challenges, and again this was irrespective of the prior hormone concentration in the animals. By contrast, pre-treatment with dexamethasone reduced the expression of *IL1B* (Fig. 6A and B, $P<0.05$) and *CXCL8* (Fig. 6E and F, $P<0.05$) in EVOCs before and following challenge with LPS or *E. coli*, and *CCL5* (Fig. 6G and H, $P<0.05$) following challenge with LPS or *E. coli*, compared with the control medium.

To determine whether the lack of effect of ovarian steroids on endometrial innate immune responses was unique to the EVOCs, experiments were also conducted using pure populations of endometrial cells. Endometrial

epithelial cells, stromal cells and PBMCs accumulated IL6 (Fig. 7A, B and C) and IL8 (Fig. 7D, E and F) in response to challenge with LPS for 24 h ($P<0.001$). However, pre-treatment with oestradiol or progesterone for 24 h had no significant effect on IL6 or IL8 production of each of the three cell types. However, pre-treatment with dexamethasone reduced the accumulation of IL6 in epithelial cells (Fig. 7A, $P<0.05$) and IL8 in stromal cells and PBMCs (Fig. 7E and F, $P<0.05$).

However, different concentrations of exogenous steroid could modulate the cellular responses and, hence, endometrial stromal cells were treated with a range of concentrations of oestradiol or progesterone, before challenge with LPS. Stromal cells were used for two reasons: first, stromal cells are more responsive to LPS than epithelial cells, and the increased dynamic range gave the best chance of observing an effect. Secondly, epithelial cells are lost during and after parturition exposing the underlying stromal cells to bacterial infection (Archbald *et al.* 1972). Pre-treatment of endometrial stromal cells with 1–30 pg/ml oestradiol (Fig. 8A and C) or 1–30 ng/ml progesterone (Fig. 8B and D) for 24 h, did not modulate IL6 or IL8 responses to LPS during subsequent challenges.

Figure 4 Ovarian steroid receptor (*ESR1* and *PGR*) expression in *ex vivo* organ cultures is regulated by progesterone and oestradiol but not by dexamethasone. Follicular-phase (A and C) or luteal-phase (B and D) endometrial EVOCs from the intercaruncular areas of beef heifer endometrium (contralateral horn) were treated for 48 h with the control medium or a medium containing 5 ng/ml progesterone, 3 pg/ml oestradiol or 5 ng/ml dexamethasone. The EVOC tissues were collected and total RNA was extracted for analysis of *ESR1* (A and B) and *PGR* (C and D) mRNA expression by qPCR. For follicular-phase EVOCs: control, $n=6$; oestradiol, $n=3$; progesterone, $n=3$ and dexamethasone, $n=3$. For luteal-phase EVOCs: control, $n=10$; oestradiol, $n=4$; progesterone, $n=4$ and dexamethasone, $n=6$. Data are expressed as dot plots with the horizontal bar representing the mean and vertical bars S.E.M. Data were analysed by ANOVA using Dunnett's pairwise multiple comparison *t*-test and values differ from control: $*P<0.05$, and each dot represents an individual animal.

Figure 5 Exogenous ovarian steroids do not regulate *ex vivo* organ culture responses to LPS or *Escherichia coli*. Follicular-phase (A, C and E) and luteal-phase (B, D and F) endometrial EVOCs from the intercaruncular areas of beef heifer endometrium (contralateral horn) were pre-treated for 24 h with the control medium (open bar) or a medium containing 5 ng/ml progesterone (chequered bar), 3 pg/ml oestradiol (striped bar) or 5 ng/ml dexamethasone (closed bar). After 24 h, the EVOCs were challenged with the control medium or a medium containing 1 µg/ml LPS or 1×10^3 CFU/ml *E. coli* for a further 24 h in the presence of the steroids. Supernatants were collected and analysed for IL1β (A and B), IL6 (C and D) and IL8 (E and F) by ELISA. Data are expressed as mean (s.e.m.). Data were analysed by GLM multivariate ANOVA and Dunnett's pairwise multiple comparison *t*-test and values differ between groups: * $P < 0.05$. The number of animals (*n*) is indicated in the figure.

Ovarian steroid receptor antagonists and endometrial responses to LPS or *E. coli*

To examine whether steroid nuclear receptor function modulates endometrial innate immunity, EVOCs were pre-treated with the ERα antagonist MMP or the PR/GR antagonist mifepristone, with or without the appropriate steroid present. After the 24 h pre-treatment, EVOCs were challenged with the control medium or a medium containing LPS or *E. coli*. Endometrial EVOCs accumulated IL1β, IL6 and IL8 in response to challenge with LPS and IL1β and IL6 following challenge with *E. coli* (Fig. 9, $P < 0.0001$). Pre-treatment of EVOCs for 24 h with oestradiol and/or MMP had no effect on endometrial responses to challenge with LPS or *E. coli* (Fig. 9A, D and G). Pre-treatment for 24 h with progesterone and/or mifepristone also had no significant effect on endometrial responses to challenge with LPS or *E. coli* (Fig. 9B, E and H). However, pre-treatment with dexamethasone reduced ($P < 0.05$) the accumulation of

IL1β in response to challenge with *E. coli*, and importantly, pre-treatment with dexamethasone and mifepristone blocked the IL1β-inhibiting effect of dexamethasone (Fig. 9C, $P < 0.05$).

Discussion

In vivo, there is clear evidence for a protective effect of oestradiol or oestrus against infection of the uterus, and for a disease-promoting effect of progesterone or the luteal phase of the oestrus cycle (Rowson *et al.* 1953, Del Vecchio *et al.* 1992, Lewis 2004). Although, several

Figure 6 *Ex vivo* organ culture mRNA expression is regulated by dexamethasone, but not by the ovarian steroids. Follicular-phase (A, C, E and G) or luteal-phase (B, D, F and H) endometrial EVOCs from the intercaruncular areas of beef heifer endometrium (contralateral horn) were pre-treated for 24 h with the control medium (closed circle) or a medium containing 5 ng/ml progesterone (open triangle), 3 pg/ml oestradiol (open square) or 5 ng/ml dexamethasone (closed square). The EVOCs were then challenged with the control medium or a medium containing 1 µg/ml LPS or 1×10^3 CFU/ml *Escherichia coli* for a further 24 h in the presence of the steroids. The EVOC tissues were collected and total RNA was extracted for analysis of *IL1B* (A and B), *IL6* (C and D), *CXCL8* (E and F) and *CCL5* (G and H) mRNA expression by qPCR. For follicular-phase EVOCs: control, *n* = 6; oestradiol, *n* = 5; progesterone, *n* = 5 and dexamethasone, *n* = 3. For luteal-phase EVOCs: control, *n* = 10; oestradiol, *n* = 6; progesterone, *n* = 6 and dexamethasone, *n* = 4. Data are expressed as dot plots with the horizontal bar representing the mean and vertical bars s.e.m. Data were analysed by GLM multivariate ANOVA and Dunnett's pairwise multiple comparison *t*-test, and values differ between groups: * $P < 0.05$, and each dot represents an individual animal.

Figure 7 Ovarian steroids do not regulate endometrial cell responses to LPS. Endometrial epithelial (A and D) and stromal (B and E) cells from the ipsilateral horn of early-luteal-phase beef heifer endometrium, and peripheral blood-derived mononuclear cells (PBMCs; C and F) from beef heifers were pre-treated for 24 h with the control medium (open bar) or a medium containing 5 ng/ml progesterone (chequered bar), 3 pg/ml oestradiol (striped bar) or 5 ng/ml dexamethasone (closed bar). After 24 h, cells were challenged with the control medium or a medium containing 100 ng/ml LPS for a further 24 h in the presence of the steroids. Supernatants were collected and analysed for IL6 (A, B and C) and IL8 (D, E and F) by ELISA. Data are expressed as mean (S.E.M.). Data were analysed by GLM multivariate ANOVA and Dunnett's pairwise multiple comparison *t*-test, and values differ from control: * $P < 0.05$. Number of animals, $n = 4$.

explanations for these effects have been explored previously, the mechanistic explanations are elusive, particularly in relation to leukocyte population differences and neutrophil function (Subandrio & Noakes 1997, Subandrio *et al.* 2000, Winters *et al.* 2003). Thus, we reasoned that ovarian steroids might modulate innate immune responses in the endometrium. However, in this study, the stage of the oestrus cycle did not influence the cytokine or chemokine response of *ex vivo* endometrial tissue to *E. coli* or LPS at the gene or the protein level. Furthermore, exogenous ovarian steroids did not modulate the innate immune response by endometrial tissue or cells. Finally, even blocking the nuclear receptors for oestradiol or progesterone did not affect the inflammatory response to *E. coli* or LPS. We conclude that ovarian steroids have little effect on *in vitro* inflammatory responses associated with innate immunity in the bovine endometrium.

The use of EVOCs maintains the architecture of cells in the tissue and retains an imprint of the stage of the oestrus cycle of the animal. Using EVOCs also avoids potential confounders of *in vivo* studies, including humoral factors, effects of nutrition and adaptive immune responses, enabling exploration of the impact of steroids in the localised tissue and cells of the endometrium, independent of the whole-animal response. In this study, endometrial EVOCs from beef heifers were a good surrogate for tissues from dairy cows, producing similar cytokine and chemokine responses to *E. coli* and LPS. Furthermore, using tissue and cells from beef heifers, potential confounders in dairy cows, such as insemination, pregnancy, previous uterine disease and

Figure 8 High concentrations of ovarian steroids do not regulate endometrial stromal cell responses to LPS. Endometrial stromal cells from the ipsilateral horn of early-luteal-phase beef heifer endometrium were pre-treated for 24 h with the control medium or a medium containing 1–30 pg/ml oestradiol (A, C and E), 1–30 ng/ml progesterone (B, D and F) or 5 ng/ml dexamethasone. After 24 h, cells were challenged with the control medium or a medium containing 100 ng/ml LPS for a further 24 h in the presence of the steroids. Supernatants were collected and analysed for IL6 (A and B) and IL8 (C and D) by ELISA. Cell viability was determined by MTT assay (E and F). Data are expressed as mean (S.E.M.). Data were analysed by ANOVA and Dunnett's pairwise multiple comparison *t*-test, and values differ from LPS: * $P < 0.05$. Number of animals, $n = 3$.

Figure 9 Competitive inhibition of ovarian nuclear receptors within ex vivo organ cultures. Endometrial EVOCs were harvested from the intercaruncular areas of early-luteal-phase beef heifer endometrium (contralateral horn), and treated for 24 h with the control medium or a medium containing 3 pg/ml oestradiol and/or 100 nM MPP (A, D and G), 5 ng/ml progesterone and/or 100 nM mifepristone (B, E and H) or 5 ng/ml dexamethasone and/or 100 nM mifepristone (C, F and I). After 24 h, EVOCs were challenged with the control medium or a medium containing 1 µg/ml LPS or 1×10^3 CFU/ml *Escherichia coli* for a further 24 h in the presence of the steroids/antagonists. Supernatants were collected and analysed for IL1β (A, B and C), IL6 (D, E and F) and IL8 (G, H and I) by ELISA. Data are expressed as mean (S.E.M.). Data were analysed by two-way ANOVA and Dunnett's pairwise multiple comparison test, and values differ between groups: * $P < 0.05$. The number of animals (n) is indicated within the figure.

lactation, were removed. Yet, the increased cytokine and chemokine secretion and increased mRNA expression following challenge of EVOCs with *E. coli* or LPS mirror the changes during disease *in vivo* (Herath *et al.* 2009b, Sheldon *et al.* 2009). Additionally, EVOCs collected from the horn ipsilateral or contralateral to the active corpus luteum were equally responsive to LPS, suggesting that the inflammatory response is not modulated by differing concentration gradients of the hormone across the two horns. This view is supported by gene array analyses, which report hundreds of differentially expressed genes in the endometrium of luteal- vs follicular-phase animals, but very few genes differ in expression between the horn ipsilateral and contralateral to the corpus luteum, and those that do have very low ratios (Bauersachs *et al.* 2005, Shimizu *et al.* 2010). However, EVOCs incorporating tissue from intercaruncular areas

of the endometrium were more responsive to challenge with LPS or *E. coli* than caruncular tissue. With over 1100 differentially expressed genes between intercaruncular and caruncular tissues, including several inflammation and immune-regulating genes (Mansouri-Attia *et al.* 2009), use of tissues from the intercaruncular zones was an important optimisation step.

Central to the response to bacterial challenge is the detection of pathogen-associated molecular patterns by TLRs, and in particular for *E. coli* infection, binding of LPS by TLR4. Endometrial epithelial and stromal cells also express TLRs, including TLR4, and produce IL6 and IL8 following challenge with LPS (Herath *et al.* 2006, Sheldon & Roberts 2010). In this study, *E. coli* and LPS stimulated the accumulation of IL1β and IL8 by 24 h. The kinetics of IL6 production probably reflects the roles of IL6 in the early response to infection such as leukocyte

recruitment, B-lymphocyte development, antibody secretion by plasma cells and the regulation of acute-phase proteins. Interleukin 8 (IL8), a potent chemo-attractor and activator of neutrophils and T-lymphocytes, is secreted by monocytes, lymphocytes, fibroblasts and epithelial and endothelial cells (Mukaida 2000, Schaefer *et al.* 2004). IL1 β is secreted predominantly by monocytes following inflammasome activation and stimulates the production of additional pro-inflammatory cytokines, such as IL6 (van de Veerdonk *et al.* 2011), and chemokines such as IL8, which recruit more immune cells and promote phagocytosis and bacterial clearance (Petrilli *et al.* 2007).

The most striking observations in this study were that endometrial tissue and cell responses to challenge with *E. coli* or LPS were not influenced by the stage of the oestrus cycle, or by the addition of exogenous oestradiol or progesterone. First, we found that the stage of the oestrus cycle did not affect innate immune responses of endometrial EVOCs challenged with LPS or *E. coli*. Hence, we considered whether separating out the cellular populations would uncover steroid-responsive effects, using differential regulation of *ESR1* and *PGR* mRNA to verify that the endometrial cells were responsive to exogenous oestradiol and progesterone. However, ovarian steroids had no effect on separated endometrial cell or PBMC responses to challenge with LPS or *E. coli*, and similarly, inhibition of ER α or PR had no effect on innate immune responses; although, inhibiting the GR inhibited dexamethasone-related inflammatory modulation. One could argue that the initial staging of the oestrus cycle may have been erroneous. However, more than 150 animals were used across the studies, peripheral plasma progesterone concentrations were used to verify the stage of cycle, and the variance across groups was small irrespective of the stage of cycle. It could also be argued that higher steroid concentrations in the uterine tissue, compared with the peripheral plasma, might effectively modulate inflammatory responses (McCracken *et al.* 1984, Weems *et al.* 1988, Einer-Jensen *et al.* 1989). However, extended dose range experiments showed no effect on inflammatory responses to LPS, and EVOC tissue had been exposed to native uterine steroid concentrations. Taken together, these data suggest that there is neither a direct effect of the ovarian steroids on innate immunity nor oestrus cyclic regulation of ovarian steroid receptor expression likely to affect innate immunity.

In vivo, there is a clear oestrus-dependent effect on basal mRNA expression of cytokines and chemokines such as *IL1B*, *CXCL8* and *CXCL5* in cells collected from the uterine lumen (Fischer *et al.* 2010). Thus, this gives rise to a question as to how the negative results of this study *in vitro* fit into the whole animal effects? First, there may be an innate immune effect mediated by regulatory molecules not investigated in this study. *In vitro*, exogenous ovarian steroids reduce the synthesis of

prostaglandin F_{2 α} and prostaglandin E₂ in endometrial cells (Herath *et al.* 2006). Other classes of molecules, such as antimicrobial peptides, lipoxins or resolvins, could also be examined. Secondly, alteration of the adaptive immune response would have a significant effect on disease outcome. The presence of ovarian steroid receptors on immune cells suggests the possibility of their regulation, and there is evidence from humans that ovarian steroids have a significant impact on disease outcome (Waage *et al.* 1990, Wira *et al.* 2005, Rodriguez-Garcia *et al.* 2013a,b). Thirdly, there may be an indirect effect of ovarian steroids on innate or adaptive immunity. Indeed, in this study, dexamethasone reduced IL1 β , IL6 and IL8 secretion, together with *IL1B*, *IL6*, *CXCL8* and *CCL5* mRNA responses to challenge with *E. coli* or LPS. In addition, the GR antagonist mifepristone attenuated the suppressive effect of dexamethasone on IL1 β secretion following *E. coli* challenge. Kuse *et al.* have recently demonstrated a regulatory effect of ovarian steroids on *NR3C1* expression within the bovine endometrium. As a function of the stage of the oestrus cycle, *NR3C1* expression within the bovine endometrium was greater during the mid-luteal phase when progesterone concentrations are high, compared with other phases of the oestrus cycle, and the glucocorticoid cortisol more strongly suppressed PGF_{2 α} production during the mid-luteal phase than during the follicular phase. The addition of progesterone to cultured endometrial epithelial cells also increased expression of *NR3C1*, while oestradiol reduced expression levels (Kuse *et al.* 2013). A future approach might also probe single cell responses to PAMPs and steroids as, although this study has shown no response to ovarian steroids within large populations of cells *in vitro*, individual cells may respond. Indeed, such an approach has recently revealed the production of the lymphosteroid pregnenolone by Th2 T cells, which is associated with immunosuppression, inhibiting Th cell proliferation and B cell immunoglobulin class switching (Mahata *et al.* 2014).

In conclusion, there was no effect of the stage of the oestrus cycle, exogenous ovarian steroids or inhibition of their nuclear receptors on key cytokine and chemokine responses to *E. coli* or LPS in endometrial tissues or cells. The lack of effect of ovarian steroids challenges the central dogma that steroids suppress immunity across species.

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.

Funding

This work was supported by funding from the UK Biotechnology and Biological Sciences Research Council (BB/1017240/1), FAPEMIG and CNPq fellow (Conselho

Nacional de Desenvolvimento Científico e Tecnológico – grant numbers PDE 201916/2012-6 and PDE 200885/2010-3).

Acknowledgements

The authors thank James Cronin for isolation and culture of PBMCs, and James Cronin and Steve Jeremiah for their assistance with ELISA.

References

- Amos MR, Healey GD, Goldstone RJ, Mahan SM, Düvel A, Schuberth HJ, Sandra O, Zieger P, Dieuzy-Labaye I, Smith DG *et al.* 2014 Differential endometrial cell sensitivity to a cholesterol-dependent cytolysin links *Trueperella pyogenes* to uterine disease in cattle. *Biology of Reproduction* **90** 54. (doi:10.1095/biolreprod.113.115972)
- Archbald LF, Schultz RH, Fahning ML, Kurtz HJ & Zemjanis R 1972 A sequential histological study of the post-partum bovine uterus. *Journal of Reproduction and Fertility* **29** 133–136. (doi:10.1530/jrf.0.0290133)
- Bauersachs S, Ulbrich SE, Gross K, Schmidt SE, Meyer HH, Einspanier R, Wenigerkind H, Vermehren M, Blum H, Sinowatz F *et al.* 2005 Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. *Journal of Molecular Endocrinology* **34** 889–908. (doi:10.1677/jme.1.01799)
- Borges AM, Healey GD & Sheldon IM 2012 Explants of intact endometrium to model bovine innate immunity and inflammation *ex vivo*. *American Journal of Reproductive Immunology* **67** 526–539. (doi:10.1111/j.1600-0897.2012.01106.x)
- Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL *et al.* 2009 The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55** 611–622. (doi:10.1373/clinchem.2008.112797)
- Cronin JG, Turner ML, Goetze L, Bryant CE & Sheldon IM 2012 Toll-like receptor 4 and MYD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. *Biology of Reproduction* **86** 51–59. (doi:10.1095/biolreprod.111.092718)
- Del Vecchio RP, Matsas DJ, Inzana TJ, Sponenberg DP & Lewis GS 1992 Effect of intrauterine bacterial infusions and subsequent endometritis on prostaglandin F_{2α} metabolite concentrations in postpartum beef cows. *Journal of Animal Science* **70** 3158–3162.
- Einer-Jensen N, McCracken JA, Schram W & Bendz A 1989 Counter current transfer in the female adnex. *Acta Physiologica Polonica* **40** 3–11.
- Fischer C, Drillich M, Odau S, Heuwer W, Einspanier R & Gabler C 2010 Selected pro-inflammatory factor transcripts in bovine endometrial epithelial cells are regulated during the oestrous cycle and elevated in case of subclinical or clinical endometritis. *Reproduction, Fertility, and Development* **22** 818–829. (doi:10.1071/RD09120)
- Fortier M, Guilbault L & Grasso F 1988 Specific properties of epithelial and stromal cells from the endometrium of cows. *Journal of Reproduction and Fertility* **83** 239–248. (doi:10.1530/jrf.0.0830239)
- Goldstone RJ, Talbot R, Schuberth HJ, Sandra O, Sheldon IM & Smith DG 2014 Draft genome sequence of *Escherichia coli* MS499, isolated from the infected uterus of a postpartum cow with metritis. *Genome Announcements* **2** e00217. (doi:10.1128/genomeA.00217-14)
- Herath S, Fischer DP, Werling D, Williams EJ, Lilly ST, Dobson H, Bryant CE & Sheldon IM 2006 Expression and function of Toll-like receptor 4 in the endometrial cells of the uterus. *Endocrinology* **147** 562–570. (doi:10.1210/en.2005-1113)
- Herath S, Williams EJ, Lilly ST, Gilbert RO, Dobson H, Bryant CE & Sheldon IM 2009a Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. *Reproduction* **134** 683–693. (doi:10.1530/REP-07-0229)
- Herath S, Lilly ST, Fischer DP, Williams EJ, Dobson H, Bryant CE & Sheldon IM 2009b Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F_{2α} to prostaglandin E₂ in bovine endometrium. *Endocrinology* **150** 1912–1920. (doi:10.1210/en.2008-1379)
- Herath S, Lilly ST, Santos NR, Gilbert RO, Goetze L, Bryant CE, White JO, Cronin J & Sheldon IM 2009b Expression of genes associated with immunity in the endometrium of cattle with disparate postpartum uterine disease and fertility. *Reproductive Biology and Endocrinology* **7** 55. (doi:10.1186/1477-7827-7-55)
- Ireland JJ, Coulson PB & Murphree RL 1979 Follicular development during four stages of the estrous cycle of beef cattle. *Journal of Animal Science* **49** 1261–1269.
- Jimenez-Krassel F, Folger JK, Ireland JL, Smith GW, Hou X, Davis JS, Lonergan P, Evans AC & Ireland JJ 2009 Evidence that high variation in ovarian reserves of healthy young adults has a negative impact on the corpus luteum and endometrium during estrous cycles in cattle. *Biology of Reproduction* **80** 1272–1281. (doi:10.1095/biolreprod.108.075093)
- Kern JA, Lamb RJ, Reed JC, Daniele RP & Nowell PC 1988 Dexamethasone inhibition of interleukin 1β production by human monocytes. Posttranscriptional mechanisms. *Journal of Clinical Investigation* **81** 237–244. (doi:10.1172/JCI113301)
- Kimmins S & MacLaren LA 2001 Oestrous cycle and pregnancy effects on the distribution of oestrogen and progesterone receptors in bovine endometrium. *Placenta* **22** 742–748. (doi:10.1053/plac.2001.0708)
- Kuse M, Lee HY, Acosta TJ, Hojo T & Okuda K 2013 Expression of glucocorticoid receptor alpha and its regulation in the bovine endometrium: possible role in cyclic prostaglandin F_{2α} production. *Journal of Reproduction and Development* **59** 346–352. (doi:10.1262/jrd.2012-185)
- Lewis GS 2003 Role of ovarian progesterone and potential role of prostaglandin F_{2α} and prostaglandin E₂ in modulating the uterine response to infectious bacteria in postpartum ewes. *Journal of Animal Science* **81** 285–293.
- Lewis GS 2004 Steroidal regulation of uterine immune defenses. *Animal Reproduction Science* **82–83** 281–294.
- Mahata B, Zhang X, Kolodziejczyk AA, Proserpio V, Haim-Vilmsky L, Taylor AE, Hebenstreit D, Dingler FA, Moignard V, Gottgens B *et al.* 2014 Single-cell RNA sequencing reveals T helper cells synthesizing steroids *de novo* to contribute to immune homeostasis. *Cell Reports* **7** 1130–1142. (doi:10.1016/j.celrep.2014.04.011)
- Mansouri-Attia N, Aubert J, Reinaud P, Giraud-Delville C, Taghouti G, Galio L, Everts RE, Degrelle S, Richard C, Hue I *et al.* 2009 Gene expression profiles of bovine caruncular and intercaruncular endometrium at implantation. *Physiological Genomics* **39** 14–27. (doi:10.1152/physiolgenomics.90404.2008)
- McCracken JA, Schramm W & Einer-Jensen N 1984 The structure of steroids and their diffusion through blood vessel walls in a counter-current system. *Steroids* **43** 293–303. (doi:10.1016/0039-128X(84)90047-3)
- Mosmann T 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65** 55–63. (doi:10.1016/0022-1759(83)90303-4)
- Mukaida N 2000 Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. *International Journal of Hematology* **72** 391–398.
- Nolan T, Hands RE & Bustin SA 2006 Quantification of mRNA using real-time RT-PCR. *Nature Protocols* **1** 1559–1582. (doi:10.1038/nprot.2006.236)
- Petrilli V, Dostert C, Muruve DA & Tschopp J 2007 The inflammasome: a danger sensing complex triggering innate immunity. *Current Opinion in Immunology* **19** 615–622. (doi:10.1016/j.coi.2007.09.002)
- Price JC, Bromfield JJ & Sheldon IM 2013 Pathogen-associated molecular patterns initiate inflammation and perturb the endocrine function of bovine granulosa cells from ovarian dominant follicles via TLR2 and TLR4 pathways. *Endocrinology* **154** 3377–3386. (doi:10.1210/en.2013-1102)
- Rinaldi M, Cecilian F, Lecchi C, Moroni P & Bannerman DD 2008 Differential effects of α1-acid glycoprotein on bovine neutrophil respiratory burst activity and IL-8 production. *Veterinary Immunology and Immunopathology* **126** 199–210. (doi:10.1016/j.vetimm.2008.07.001)
- Rodriguez-Garcia M, Biswas N, Patel MV, Barr FD, Crist SG, Ochsenbauer C, Fahey JV & Wira CR 2013a Estradiol reduces susceptibility of CD4+ T cells and macrophages to HIV-infection. *PLoS ONE* **8** e62069. (doi:10.1371/journal.pone.0062069)
- Rodriguez-Garcia M, Patel MV & Wira CR 2013b Innate and adaptive anti-HIV immune responses in the female reproductive tract. *Journal of Reproductive Immunology* **97** 74–84. (doi:10.1016/j.jri.2012.10.010)

- Roth JA, Kaeberle ML, Appell LH & Nachreiner RF 1983 Association of increased estradiol and progesterone blood values with altered bovine polymorphonuclear leukocyte function. *American Journal of Veterinary Research* **44** 247–253.
- Rowson LE, Lamming GE & Fry RM 1953 Influence of ovarian hormones on uterine infection. *Nature* **171** 749–750. (doi:10.1038/171749a0)
- Schaefer TM, Desouza K, Fahey JV, Beagley KW & Wira CR 2004 Toll-like receptor (TLR) expression and TLR-mediated cytokine/chemokine production by human uterine epithelial cells. *Immunology* **112** 428–436. (doi:10.1111/j.1365-2567.2004.01898.x)
- Scully S, Evans AC, Duffy P & Crowe MA 2014 Characterization of follicle and CL development in beef heifers using high resolution three-dimensional ultrasonography. *Theriogenology* **81** 407–418. (doi:10.1016/j.theriogenology.2013.10.012)
- Seals RC, Wulster-Radcliffe MC & Lewis GS 2002 Modulation of the uterine response to infectious bacteria in *postpartum* ewes. *American Journal of Reproductive Immunology* **47** 57–63. (doi:10.1034/j.1600-0897.2002.10062.x)
- Sheldon IM & Bromfield JJ 2011 Innate immunity in the human endometrium and ovary. *American Journal of Reproductive Immunology* **66** 63–71. (doi:10.1111/j.1600-0897.2011.01034.x)
- Sheldon IM & Roberts MH 2010 Toll-like receptor 4 mediates the response of epithelial and stromal cells to lipopolysaccharide in the endometrium. *PLoS ONE* **5** e12906. (doi:10.1371/journal.pone.0012906)
- Sheldon IM, Noakes DE, Rycroft AN, Pfeiffer DU & Dobson H 2002 Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. *Reproduction* **123** 837–845. (doi:10.1530/rep.0.1230837)
- Sheldon IM, Cronin J, Goetze L, Donofrio G & Schuberth HJ 2009 Defining *postpartum* uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. *Biology of Reproduction* **81** 1025–1032. (doi:10.1095/biolreprod.109.077370)
- Sheldon IM, Rycroft AN, Dogan B, Craven M, Bromfield JJ, Chandler A, Roberts MH, Price SB, Gilbert RO & Simpson KW 2010 Specific strains of *Escherichia coli* are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. *PLoS ONE* **5** e9192. (doi:10.1371/journal.pone.0009192)
- Shimizu T, Krebs S, Bauersachs S, Blum H, Wolf E & Miyamoto A 2010 Actions and interactions of progesterone and estrogen on transcriptome profiles of the bovine endometrium. *Physiological Genomics* **42A** 290–300. (doi:10.1152/physiolgenomics.00107.2010)
- Siemieniuch MJ, Bowolaksono A, Skarzynski DJ & Okuda K 2010 Ovarian steroids regulate prostaglandin secretion in the feline endometrium. *Animal Reproduction Science* **120** 142–150. (doi:10.1016/j.anireprosci.2010.02.020)
- Skinner DC, Bouchard P & Caraty A 1999 The progesterone blockade of the luteinizing hormone surge is overcome by RU486. *Journal of Neuroendocrinology* **11** 637–641. (doi:10.1046/j.1365-2826.1999.00375.x)
- Steinman RM & Cohn ZA 1973 Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *Journal of Experimental Medicine* **137** 1142–1162. (doi:10.1084/jem.137.5.1142)
- Subandrio A & Noakes D 1997 Neutrophil migration into the uterine lumen of the cow: the influence of endogenous and exogenous sex steroid hormones using two intrauterine chemoattractants. *Theriogenology* **47** 825–835. (doi:10.1016/S0093-691X(97)00038-1)
- Subandrio AL, Sheldon IM & Noakes DE 2000 Peripheral and intrauterine neutrophil function in the cow: the influence of endogenous and exogenous sex steroid hormones. *Theriogenology* **53** 1591–1608. (doi:10.1016/S0093-691X(00)00300-9)
- Sun J, Huang YR, Harrington WR, Sheng S, Katzenellenbogen JA & Katzenellenbogen BS 2002 Antagonists selective for estrogen receptor alpha. *Endocrinology* **143** 941–947.
- Turner ML, Healey GD & Sheldon IM 2012 Immunity and inflammation in the uterus. *Reproduction in Domestic Animals* **47** (Suppl 4) 402–409. (doi:10.1111/j.1439-0531.2012.02104.x)
- Turner ML, Cronin JG, Healey GD & Sheldon IM 2014 Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides *in vitro* via Toll-like receptors TLR2, TLR1 and TLR6. *Endocrinology* **155** 1453–1465. (doi:10.1210/en.2013-1822)
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** RESEARCH0034. (doi:10.1186/gb-2002-3-7-research0034)
- van de Veerdonk FL, Netea MG, Dinarello CA & Joosten LA 2011 Inflammasome activation and IL-1 β and IL-18 processing during infection. *Trends in Immunology* **32** 110–116. (doi:10.1016/j.it.2011.01.003)
- Waage A & Bakke O 1988 Glucocorticoids suppress the production of tumour necrosis factor by lipopolysaccharide-stimulated human monocytes. *Immunology* **63** 299–302.
- Waage A, Slupphaug G & Shalaby R 1990 Glucocorticoids inhibit the production of IL6 from monocytes, endothelial cells and fibroblasts. *European Journal of Immunology* **20** 2439–2443. (doi:10.1002/eji.1830201112)
- Wathes DC, Cheng Z, Chowdhury W, Fenwick MA, Fitzpatrick R, Morris DG, Patton J & Murphy JJ 2009 Negative energy balance alters global gene expression and immune responses in the uterus of *postpartum* dairy cows. *Physiological Genomics* **39** 1–13. (doi:10.1152/physiolgenomics.00064.2009)
- Weems CW, Lee CN, Weems YS & Vincent DL 1988 Distribution of progesterone to the uterus and associated vasculature of cattle. *Endocrinologia Japonica* **35** 625–630. (doi:10.1507/endocrj.1954.35.625)
- Williams EJ, Fischer DP, Noakes DE, England GC, Rycroft A, Dobson H & Sheldon IM 2007 The relationship between uterine pathogen growth density and ovarian function in the *postpartum* dairy cow. *Theriogenology* **68** 549–559. (doi:10.1016/j.theriogenology.2007.04.056)
- Winters KR, Meyer E, Van Merris VM, Van Den Broeck WL, Duchateau L & Burvenich C 2003 Sex steroid hormones do not influence the oxidative burst activity of polymorphonuclear leukocytes from ovariectomized cows *in vitro*. *Steroids* **68** 397–406. (doi:10.1016/S0039-128X(03)00040-0)
- Wira CR, Fahey JV, Sentman CL, Pioli PA & Shen L 2005 Innate and adaptive immunity in female genital tract: cellular responses and interactions. *Immunological Reviews* **206** 306–335. (doi:10.1111/j.0105-2896.2005.00287.x)
- Wulster-Radcliffe MC, Seals RC & Lewis GS 2003 Progesterone increases susceptibility of gilts to uterine infections after intrauterine inoculation with infectious bacteria. *Journal of Animal Science* **81** 1242–1252.

Received 29 April 2014

First decision 11 June 2014

Revised manuscript received 12 September 2014

Accepted 22 September 2014