Mechanisms underlying the effect of infection and inflammation on ovarian health.

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Mechanisms underlying the effect of infection and inflammation on ovarian health

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Submitted to Swansea University in fulfilment of the requirements for the Degree of Doctor of Philosophy

July 2013

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Summary

Bacterial contamination of the female genital tract after parturition is common and subsequent infections are associated with infertility. Infections of the uterus or mammary gland in cattle perturb ovarian function, with decreased dominant follicle growth rate and delayed ovulation. Granulosa cells that line ovarian follicles express the molecular machinery necessary to respond to common bacterial pathogen-associated molecular patterns (PAMPs), suggesting that granulosa cells may play a role in ovarian innate immunity.

In the present thesis, the effect of bacterial PAMPs on ovarian follicle cells was examined during follicular growth, from emergence through dominance, and the early stages of embryogenesis. Granulosa cells from emerged or dominant bovine follicles increased production of inflammatory mediators, such as IL-6 and IL-8, through Toll-like receptor (TLR)-dependent pathways in response to the PAMPs lipopolysaccharide and the synthetic triacylated lipopeptide Pam3CSK4. The requirement for TLR2 and TLR4 was confirmed by using siRNA, showing activation of MAPK intracellular pathways and inhibiting key signals (p38, MEK or NFκB) in TLR pathways. The endocrine function of granulosa cells was perturbed by PAMPs, with decreased oestradiol and progesterone output. In addition, the endocrine environment affected granulosa cellular responses, with high EGF increasing the cellular response to PAMPs. Bacterial PAMPs also perturbed the oocyte, with increased cumulus expansion and parthenote cleavage rate, as well as changes in key genes involved in oocyte maturation. Finally, a human granulosa cell line was used to examine if PAMPs perturb human as well as bovine ovarian health. Indeed, human cells also expressed TLRs and mounted a cellular response to PAMPs at the mRNA level.

This thesis provides a molecular mechanism for the perturbation of ovarian function by an infection at a site distal to the ovary.

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Acknowledgements

Firstly, I would like to thank my supervisor Professor Martin Sheldon, whose advice and support has been invaluable throughout my research. I have truly appreciated the time he gave during the last 4 years and the effort he put into helping make this thesis a success.

I enjoyed working with the team in the laboratory and would not have been able to finish my graduate studies without their technical expertise and comradeship. In particular, I would like to thank Dr James Cronin and Dr Gareth Healey for their advice on molecular techniques, Dr Cathy Thornton for her help with FACS and Dr John Bromfield for his guidance on ovarian cell culture and oocyte manipulation. I would also like to thank Sîan, Laura, Ruth and Helen for their support and friendship throughout the last 4 years.

Without financial assistance, this research would not have been possible, thus I would like to acknowledge BBSRC and Zoetis (formerly Pfizer Animal Health) for funding my PhD. I am also grateful to everyone in the Regulatory Affairs team at Zoetis for their patience and support whilst writing up the thesis and trying to converse in German. My placement with Zoetis GmBH was an enjoyable and stimulating experience and I am grateful for the opportunity to work in this market-leading company.

I would like to acknowledge my parents for providing my education and supporting me whilst an undergraduate. Lastly, I would like to thank my best friend and husband, Andy, without whose love, care, support and guidance, I would have been unable to complete my doctoral studies.

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Abbreviations

A4 – androstenedione
ABAM – antibiotic/antimycotic
ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACTB – beta actin gene
AMH – anti-Müllerian hormone
AMP/ cAMP – adenosine monophosphate/ cyclic AMP
ANOVA – analysis of variance
AP-1 – activator protein 1
ASC – apoptosis-associated speck-like protein containing a caspase recruitment domain
ATCC – American type culture collection
ATP – adenosine triphosphate
BBQ – black berry quencher
BCL2 – B-cell CLL/lymphoma 2 gene
Bhq1/Bhq2 – black hole dark quencher 1/2
BIRC5 – baculoviral IAP repeat-containing 5 gene also know as *Survivin*
BSA – bovine serum albumin
CASP3 – caspase 3, apoptosis-related cysteine peptidase (gene)
CCL5 – chemokine (C-C motif) ligand aka RANTES (regulated on activation, normal T cell expressed and secreted)
CD14/CD45 – cluster of differentiation 14/45
CDX2 – caudal type homeobox 2 gene
CL – corpus luteum
CO_2 – carbon dioxide
COC – cumulus oocyte complex
Ct – threshold cycle
CXCL1/CXCL2 – chemokine (C-X-C motif) ligand 1/2
CYP19A1 – cytochrome P450, family 19, subfamily A, polypeptide 1 also known as *aromatase*
DAMP – damage-associated molecular pattern
DC (assay) – detergent compatable (assay)
DMAP – 4-Dimethylaminopyridine
DMEM – Dulbecco's modified Eagle's medium
DMSO – dimethyl sulfoxide
DNA/cDNA – deoxyribonucleic acid (complementary DNA)
DNMT1 – DNA (cytosine-5)-methyltransferase 1 gene
DRAQ5 – 1, 5-bis (2-(di-methylamino)ethyl-amino)-4, 8-dihydroxyanthracene-9, 10-dione
DUSP1 – dual specificity phosphatase 1 gene
E. coli – *Escherichia coli*
E2 – oestradiol
EDTA – ethylenediaminetetraacetic acid

EGF/EFF-R/*EGFR* – epidermal growth factor/ EGF receptor /EGF-R gene
EIA – enzyme immunoassay
ELISA – enzyme-linked immunosorbent assay
ERK1/2 – extracellular-signal-regulated kinases 1/2
EU – European Union
FACS – fluorescent activates cell sorting
F-actin – filamentous actin
FAM – carboxyfluorescein
FCS – foetal calf serum (also known as foetal bovine serum FBS)
FLA – flagellin
FSH/FSH-R/*FSHR* – follicle stimulating hormone/ FSH receptor/ FSH-R gene
GAB1 – growth factor receptor bound protein 2-associated-binding protein 1 gene
GC – granulosa cell
GDF9 – growth differentiation factor 9 gene
GNRH – gonadotrophin-releasing hormone
GVBD – germinal vesicle breakdown
H19 – imprinted maternally expressed transcript (non-protein coding)
H_2O_2 – hydrogen peroxide
H_2SO_4 – sulphuric acid
HA/ULMW HA/LMW HA/HMW HA – hyaluronic acid also known as hyaluronan/ultra-low molecular weight HA, low MW HA, high MW HA
HAS2 – hyaluronan synthase 2 gene
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEX – 6-carboxy-2 ,4,4 ,5 ,7,7 -hexachlorofluorescein succinimidyl ester
HMGB1 – high mobility group box 1
HMG-CoA – 3-hydroxy-3-methylglutaryl-coenzyme A
HRP – horse-radish peroxidise
HSP – heat shock protein
IFN – interferon
IGF(1)/IGFBP – insulin-like growth factor (1)/ IGF binding protein
IgG – immunoglobulin G
IkBa//IKK(i) – NFκB inhibitor alpha/ IκB kinase (inhibitor)
IL-(6) – interleukin (6)
Il6ST – interleukin 6 signal transducer (gp130, oncostatin M receptor) gene
IRAK(1) – interleukin-1 receptor-associated kinase (1)
IRF(3) – interferon regulatory factor (3)
IVF – *in vitro* fertilisation
IVM – *in vitro* maturation
JGCT – juvenile granulosa cell tumour
JNK – c-Jun N-terminal kinase
L19 – ribosomal protein L19 gene also known as *RPL919*
LH/LH-R/*LHCGR* – lutinising hormone/ LH receptor/ LH-R gene
LPS – lipopolysaccharide

LTA – lipoteichoic acid
LY96 – lymphocyte antigen 96 also known as MD-2
M199 – medium 199
MAP(K) – mitogen-activated protein (kinases)
MEK – dual specificity mitogen-activated protein kinase kinase
MHCII – major histocompatability complex II
min – minutes
MNC – mononuclear cells
MPF – maturation promoting factor
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88 – myeloid differentiation primary response gene 88
N_2 – nitrogen
Na_3CO_3 – sodium carbonate
NA_2HPO_4 – sodium phosphate dibasic
NaCl – sodium chloride
NaH_2PO_4 – monosodium phosphate
$NaHCO_3$ – sodium bicarbonate
NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP(3) – NOD-like receptor family, pyrin domain containing 3
NLRP5 – NLRP5 gene also known as *MATER*
NURR1 – nuclear receptor related 1 protein gene
O_2 – oxygen
p38 MAPK – p38 MAP Kinase
P4 – progesterone
PAM – Pam3CSK4
PAMP – pathogen-associated molecular pattern
PBMC – peripheral blood mononuclear cell
PBS – Dulbecco's phosphate buffered saline
PCR – polymerase chain reaction
$PG/PGE_2/PGF_{2a}$ – prostaglandin E2/F2alpha
PGN – peptidoglycan
PID – pelvic inflammatory disease
PIP_2 – phosphatidylinositol 4,5-bisphosphate
PKA – protein kinase A
PKC – protein kinase C
PLAC8 – placenta-specific 8 gene
POU5F1 – POU class 5 homeobox 1 gene also known as *Oct4*
PMA – phorbol 12-myristate 13-acetate
PTGS2 – prostaglandin-endoperoxide synthase 2 gene
PTX3 – pentraxin 3, long gene
PVDF – polyvinylidene fluoride
(t)(m)RNA/RNase – (transfer) (messenger) ribonucleic acid/ ribonuclease
RT – reverse transcriptase
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

sec – seconds
SEM – standard error of the mean
siRNA – short interfering RNA
SMAD3 – mothers against decapentaplegic homolog 3
STAT(3) – signal transducer and activator of transcription 3
T pyogenes – *Trueperella pyogenes* (also known as *Arcanobacterium pyogenes*)
TAE – Tris/acetic acid/EDTA buffer
Tak1 – TGF-beta activated kinase 1
Tbk1 – tank-binding kinase 1 (serine/threonine protein kinase)
TBS/T – Tris-buffered saline/tween
TE – Tris/EDTA buffer
TGFβ – transforming growth factor beta
TIR domain – Toll/interleukin-1 receptor (TIR) homology domain
TIRAP – TIR domain containing adapter protein
TLR – Toll-like receptor
TMB – 3,3',5,5'-tetramethylbenzidine
TNF – tumour necrosis factor
TRAF(6) – TNF receptor associated factor (6)
TRAM – TRIF-related adaptor molecule
TRIF – TIR-domain-containing adapter-inducing interferon-β
Tyr – tyrosine
TZP – transzonal projections
ZAR1 – zygote arrest 1 gene

Publications arising from the work presented in the present thesis

Chapter 3

Price JC and Sheldon IM (2013). Granulosa cells from emerged antral follicles of the bovine ovary initiate inflammation in response to bacterial pathogen-associated molecular patterns via Toll-like receptor pathways. *Biology of Reproduction* (accepted September 2013)

Chapter 4

Price JC, Bromfield JJ and 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

Chapter 6

Price JC, Cronin J and Sheldon IM (2012). Toll-like receptor expression and function in the COV434 granulosa cell line. *American Journal of Reproductive Immunology* 68; 205-217

Chapter 1

Introduction

1.1 General introduction

Ovarian health is central to successful reproduction. Microbial infections of the reproductive tract can cause uterine disease, which causes sub-fertility. Uterine disease is common in cattle and humans and, in addition to causing pain and suffering to the individual, the disease is also an economic burden for society. Early research into uterine disease focussed on visible symptoms and epidemiology, with more recent investigations beginning to address the underlying mechanisms leading to infertility. Studies have tended to examine the effect of bacterial infections on the uterus, but the disease also impacts the ovaries *in vivo*. This thesis will focus on how bacterial infections distant to the ovaries impact the function of cells within ovarian follicles.

As uterine disease and the associated ovarian dysfunction have principally been identified in dairy cattle, much of the research uses bovine ovaries. Ovarian cells and oocytes are readily available from healthy animals after slaughter. The first three research chapters address mechanisms of inflammation and immunity in the bovine ovary. The bovine ovary is also a good model for the human ovary, as it is similar in size and function. Obtaining healthy human ovaries is near impossible as most people having an oophorectomy will either have pre-existing ovarian problems or will be post-menopausal. Thus, in the penultimate chapter of the thesis, results are presented from investigations using a human ovarian cell line.

In the present chapter, the background literature to the thesis will be discussed. It will start by concentrating on the reproductive system, describing bovine ovarian structure and function, before reviewing follicular growth, ovulation and early embryogenesis. The focus of the literature review will then shift to centre on uterine disease, the associated bacterium *Escherichia coli* and how the body responds to pathogen invasion. The two sides of the story will then be linked by describing the interactions between endocrine and innate immune pathways. Lastly, the culture techniques previously used will be reviewed and the aims and objectives of this thesis identified.

1.2 Ovarian structure and function

Ovaries are gonads of female mammals, which have two main functions: to produce oocytes with the capacity for fertilisation and development, and to act as endocrine glands, secreting steroid hormones necessary for establishment of pregnancy. Each healthy ovary is around 3.5 cm x 2.5 cm x 1.5 cm and is covered in a monolayer of flat-to-cuboidal epithelium, except at the hilum where mesentery attaches to suspend the ovary from the back of the broad ligament of the uterus, as shown in Fig 1.1 (Auersperg et al., 2001). The inside of the ovary is divided into cortex and medulla, with the outer part of the cortex forming the tunica albuginea, a thin connective tissue layer. The remaining cortex contains stromal cells, capillaries, follicles and corpus lutea. Of main interest to this thesis are the granulosa cells (GCs) contained within the basement membrane of ovarian follicles.

Bovine ovaries develop from gonadal primordia as a paired thickening of the coelomic epithelium on the ventral-medial surface of the urogenital ridge on around day thirty of embryo development (Wrobel and Suss, 1998). Primordial germ cells, the embryonic pre-cursors of adult gametes, are necessary for formation of the ovary: in their absence, the gonad degenerates into a seminiferous cord-like structure (Whitworth, 1998). These germ cells originate from the hindgut and migrate through the dorsal mesentery to the gonad, where they rapidly proliferate. From this point, mitosis, meiosis and atresia of primordial oocytes (oogonia) occur, with the rate of mitosis decreasing and the rate of atresia increasing at a similar rate, until mitosis ends at 150 days (Erickson, 1966). Oogenesis is the development of meiotic oocytes from mitotic oogonia. Bovine oogonia initiate meiosis 75-80 days post conception, occurring in a wave from the anterior to the posterior side of the ovary. After the first round of cell division, premeiotic DNA is synthesised, maternal and paternal chromatids exchange genetic information and the oogonium develops into an oocyte. The oocyte is arrested at the final stage of prophase when somatic pregranulosa cells enclose the oocyte, forming primordial follicles and the oocyte secretes coat proteins to form the zona pellucida. The first meiotic division is completed at ovulation and the oocyte proceeds to the second meiotic division but is arrested at metaphase until fertilisation.

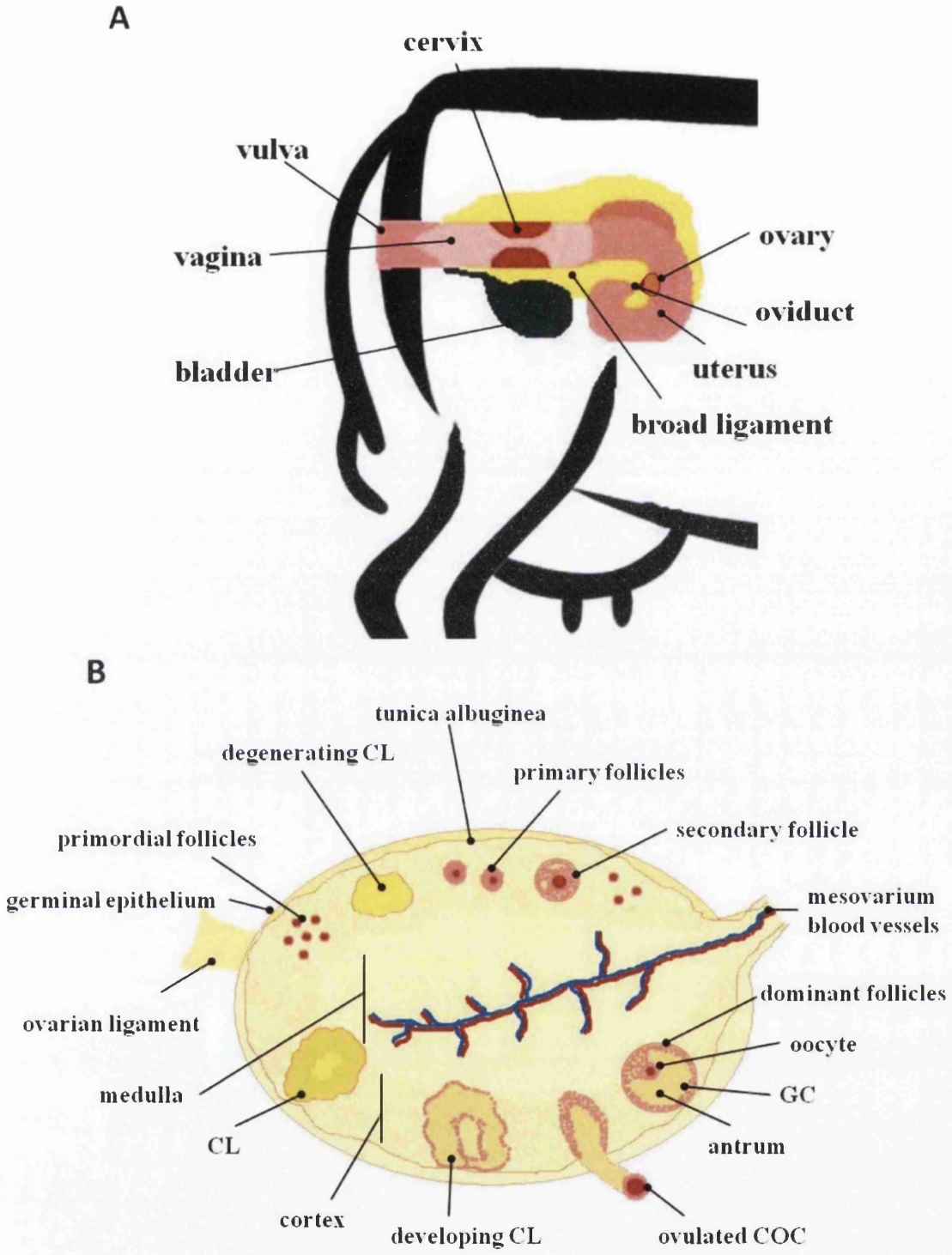


Figure 1.1 Structure and location of ovaries in the cow. (A) Bovine ovaries are suspended near the end of the oviducts and are located near the end of the uterine horn. (B) Structure of the ovary showing a developing follicle. CL – corpus luteum; GC – granulosa cell; COC – cumulus-oocyte complex

1.3 Reproductive hormones

Female reproduction is controlled by the timely release of gonadotrophins and steroid hormones. The pattern of release will be described below, but first it is important to have an understanding of how these chemicals are formed and their basic function. Steroids are terpenoid lipids, synthesised via the HMG-CoAR (3-hydroxy-3-methyl-glutaryl coenzyme A reductase) pathway (Davidson and Sittman, 1999). Steroidogenesis is the process by which steroids are formed by transformations of other steroids, leading to a pathway of steroid synthesis (Fig 1.2). The major female steroid hormones are oestrogens and progesterones and are mainly produced by the ovaries. The gonadotrophins (glycoprotein hormones) follicle-stimulating hormone (FSH) and luteinising hormone (LH) are secreted by the anterior pituitary gland in response to gonadotrophin-releasing hormone (GnRH) and act on the ovaries after transport through the circulatory system. High-frequency pulses of GnRH result in release of LH, with low-frequency pulses resulting in FSH release (Wildt et al., 1981, Kaiser et al., 1997). Prostaglandins (PG) are produced by cyclooxygenase degradation of arachidonic acid, and are secreted by the uterus to influence the corpus luteum, either promoting ($PGF_{2\alpha}$) or protecting from (PGE_2) luteolysis.

Steroids are synthesised from cholesterol, which is imported into theca cells mainly through the low-density lipoprotein (LDL) receptor, where it is maintained within lipid droplets. A hydrolase enzyme converts this cholesterol into free cholesterol, which is internalised into mitochondria through the rate-limiting action of steroidogenic acute regulatory protein (StAR). Here, the cholesterol is converted into pregnenolone, which diffuses out of the mitochondria. Through a chain of enzymatic events, pregnenolone is converted into progesterone, androstenedione and testosterone. Androstenedione and, to a lesser extent, testosterone are utilised by GCs to produce 17β-oestradiol, the main oestrogen produced by bovine ovaries. Progesterone is produced mainly by the corpus luteum after ovulation, with low plasma concentrations prior to ovulation at around 0.25 ng/ml, rising to 7 ng/ml whilst a corpus luteum is present (Malhi et al., 2005).

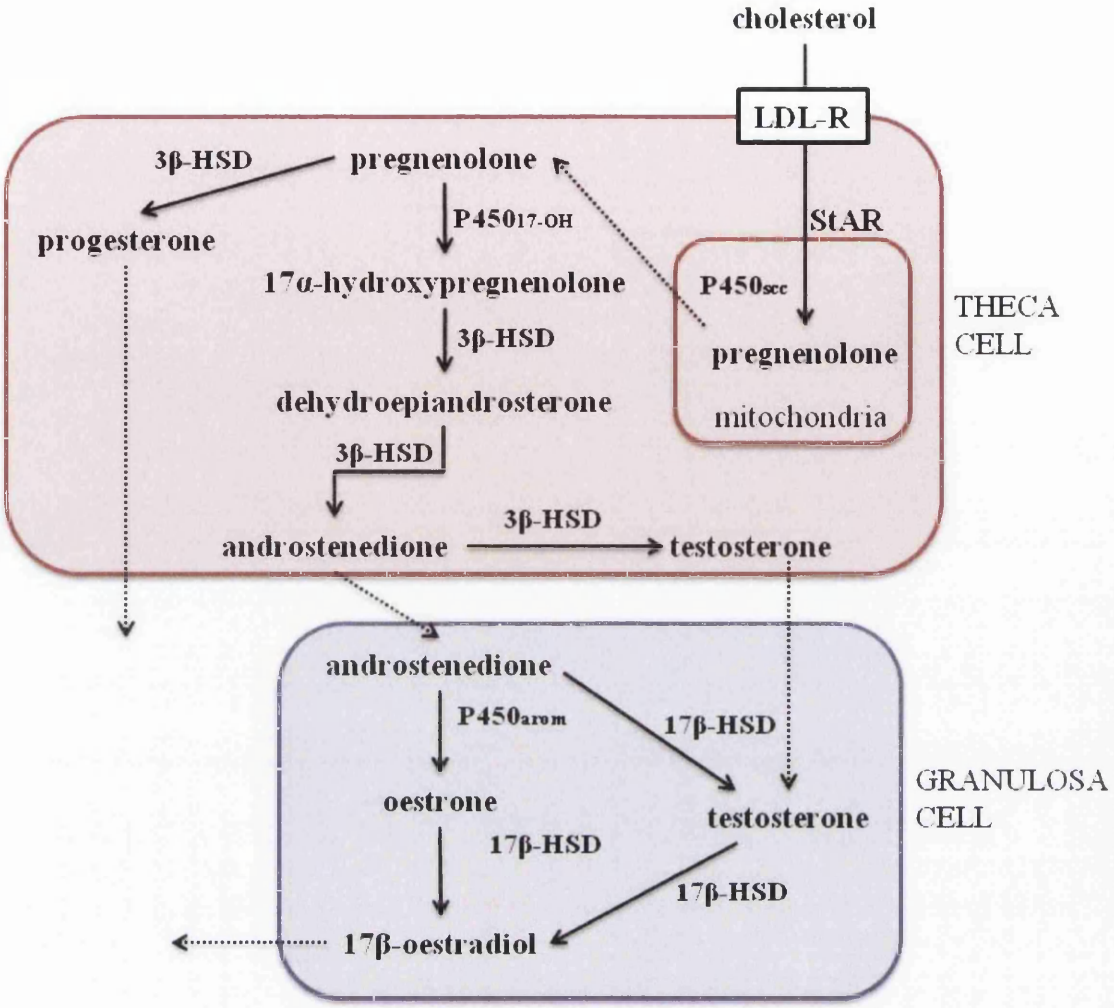


Figure 1.2 Steroid hormones are produced through an enzyme cascade which begins with cholesterol. In the ovaries, oestradiol is produced by GCs, through aromatase oxidation and elimination of a methyl group of androstenedione, released from the theca cells. LDL-R: low density lipoprotein receptor, StAR: steroidogenic acute regulatory protein, $P450_{scc}$: cholesterol side-chain cleavage enzyme, $P450_{17\text{-}OH}$: 17α-hydroxylase, 3β-HSD: 3β-hydroxysteroid dehydrogenase, $P450_{arom}$: aromatase

1.4 Oestrous cycle

The oestrous cycle consists of cyclic release of steroids (Fig 1.3), mainly 17β-oestradiol prior to ovulation and progesterone after ovulation, corresponding to physical changes in the body and in female behaviour. The bovine oestrous cycle lasts for 21 days (Stabenfeldt et al., 1969) and can be divided into four phases: oestrus, proestrus, metoestrus and dioestrus (Ball and Peters, 2004). Oestrus is designated as the first day of a new cycle and is characterised by the dominant follicle reaching its maximal size and ovulation occurring. Ovulation is induced by a preovulatory peak in concentrations of LH and occurs approximately thirty hours after the onset of oestrus (Kaim et al., 2003, Fortune et al., 2009). In the absence of gonadotrophins, oestrus does not occur (Crowe et al., 2001). During this time, circulating concentrations of FSH and oestrogen decline, and concentrations of progesterone begin to increase.

At the end of oestrus, metoestrus occurs and lasts for around 3 days. A corpus luteum is formed which secretes progesterone. During metoestrus, bleeding may occur with streaks of blood present in the vaginal mucus. This bleeding is not indicative of a failure to conceive or of conception and is not menstruation (Ball and Peters, 2004). Dioestrus occurs after metoestrus and persists for twelve days, during which time, the corpus luteum is at maximum size coinciding with high concentrations of progesterone. During dioestrus, follicles continue to grow, but until luteolysis, ovulation is unable to occur, due to the inhibitory effect of high progesterone on the release of LH.

If pregnancy has not occurred, the uterus releases $PGF_{2\alpha}$ which causes luteolysis. During proestrus, the corpus luteum recedes and plasma progesterone concentrations decline to basal concentrations. A new dominant follicle is selected, which leads to a high circulatory concentration of oestradiol (Allrich, 1994). The low concentration of progesterone combined with the high concentration of oestradiol triggers the peak of LH that precedes ovulation. Oestrus is induced by the action of oestradiol on the hypothalamus, occurring with a lack of progesterone due to luteolysis of the CL. Alternatively, if pregnancy has occurred, the embryo releases interferon tau, preventing the release of $PGF_{2\alpha}$ and thus maintaining high concentrations of progesterone.

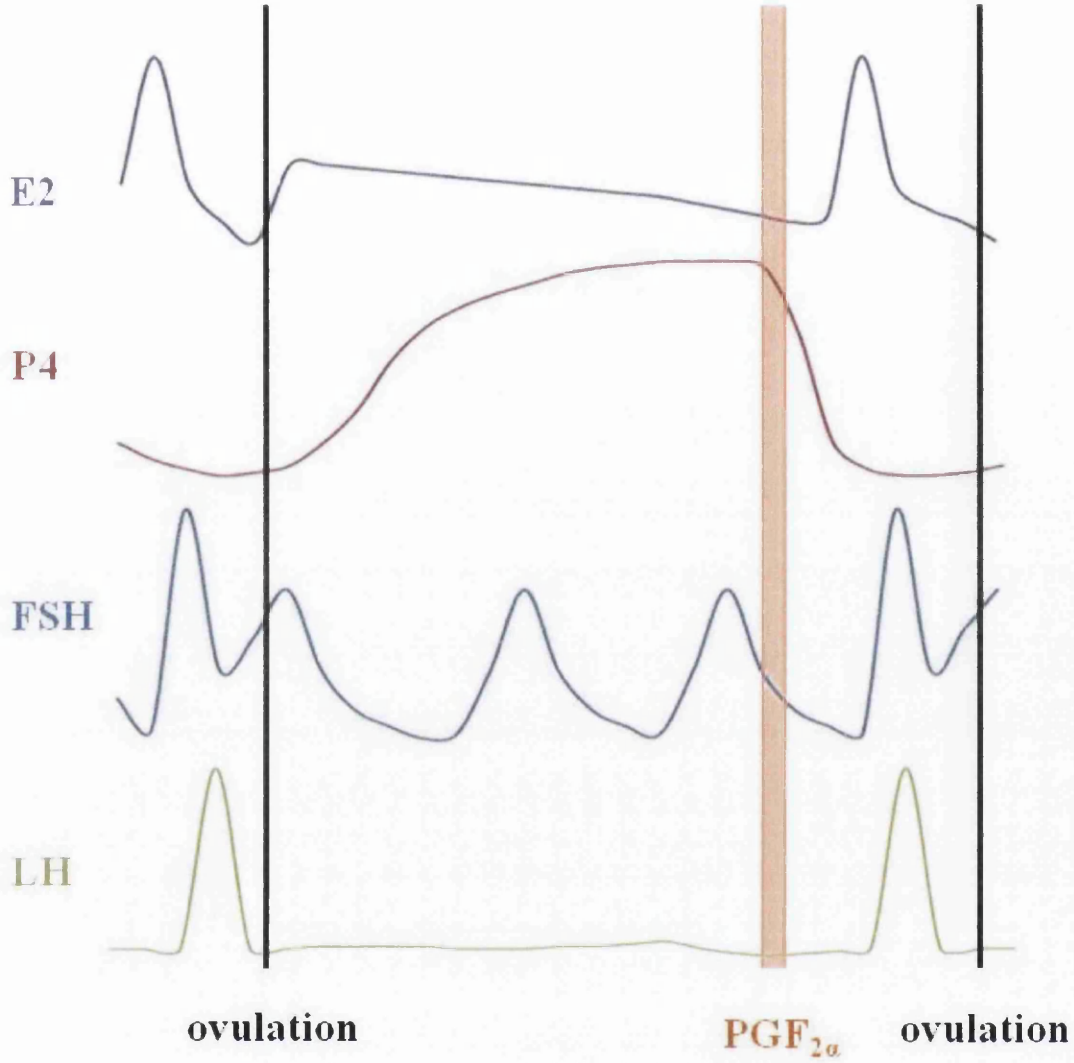


Figure 1.3 Hormone dynamics of the oestrous cycle. The graph starts with ovulation of the dominant follicle, after which there is a rise in FSH concentrations, triggering growth of the next wave of follicles. Whilst a corpus luteum is present, progesterone concentrations remain high, preventing a further ovulation. If pregnancy has not occurred, the uterus releases PGF_{2a}, which causes luteolysis, thus progesterone concentrations drop and ovulation is triggered.

1.5 Follicle development

Follicles are the functional units of the ovary, with each follicle consisting of an oocyte surrounded by layers of somatic GCs. The cells within the follicle are separated from interstitial tissue by a basal lamina, thus neither the oocyte nor GCs have a vascular supply. Gap junctions connect the cytoplasm of adjacent cells, allowing exchange of second messengers, metabolites and electrical communication between cells. As the follicle grows, a layer of theca cells outside the basal lamina is formed and becomes vascularised. Increasing layers of mural GCs are formed within the follicle, around a fluid-filled antrum. Cumulus GCs surround and nurture the oocyte.

Follicular development is a complex process (Fig 1.4) that starts before birth and takes around 180 days, from 30 μM at the primordial stage (Wandji et al., 1996) to 12-18 mm at ovulation (Perry et al., 2007, Giordano et al., 2013). Each ovary has around 133,000 primordial follicles at birth (Erickson, 1966), each consisting of an oocyte surrounded by a single layer of flattened pregranulosa cells. Recruitment of primordial follicles into primary follicles is thought to be triggered by signalling between the oocyte and the surrounding GCs, and between granulosa and theca cells (Braw-Tal, 2002). At this stage, the pregranulosa cells of the primordial follicle transform their shape, becoming cuboidal, and the oocyte grows and acquires a zona pellucida (Smitz and Cortvrindt, 2002).

The next stage in follicle development is the transition from primary follicle to preantral or secondary follicle. This stage is marked by the growth of a second layer of GCs around the oocyte, which develop into further layers through this preantral development due to high mitotic activity of the GCs (van den Hurk and Zhao, 2005). The oocyte grows further and theca cells are recruited to surround the basal lamina. These theca cells undergo differentiation to become the theca interna and theca externa and capillaries form between the two layers to provide a blood supply. At the end of the preantral stage, there are up to nine layers of GCs (Bullock et al., 2001) and the follicle attains 200 μm in diameter (Gutierrez et al., 2000). Granulosa cells start to express the receptor for follicle stimulating hormone (FSH) during the preantral stage, although preantral and early antral growth seem to be gonadotrophin-independent as follicles continue to grow to 4 mm when

gonadotrophins are inhibited (Gong et al., 1996). Follicle growth above 4 mm is gonadotrophin-dependent and involves recruitment, selection and dominance. An interesting study investigated the effects of blocking gonadotrophin-releasing hormone, and reported that even when exogenous LH was given, follicles were unable to emerge, demonstrating the need for FSH (Crowe et al., 2001).

During recruitment, GCs within early antral follicles begin to express *CYP19A1* mRNA in response to FSH, allowing oestrogen to be produced by utilising androgens produced by theca cells. The GCs also develop the ability to metabolise cholesterol to pregnenolone, but are unable to produce progesterone. Antral follicles are characterised by the development of a fluid-filled space (the antrum) adjacent to the oocyte, and the presence of a vascular supply through a mature theca cell layer (Matsuda et al., 2012). Although the process is not completely understood, fluid from the theca capillary system is thought to cross into the ovary to form the follicular fluid through a blood-follicle barrier of around 100 kDa (Clarke et al., 2006). This provides the potential for the secretion of large products within the follicle to form an osmotic gradient, drawing fluid into the follicle. Granulosa cells produce hyaluronic acid, which is hydrophilic and has been measured at a molecular weight of $2x10^6$, thus could create osmotic potential to pull fluid into the follicle (Rodgers and Irving-Rodgers, 2010). During pre-antral follicle growth, multiple small fluid-filled cavities develop, expand and combine to form a central antrum.

It is at the early antral stage that dominant follicle selection occurs from among a number of small antral follicles with differential FSH sensitivity. Atresia of small follicles occurs through apoptosis of all cells in the follicle and the oocyte. However, remaining follicles secrete oestradiol and inhibin, which negatively feedback on FSH (Kaneko et al., 1991), starving smaller follicles with fewer FSH receptors of their gonadotrophin drive. During selection of the dominant follicle, GCs within the follicle begin to express mRNA encoding the LH receptor and develop the capability to synthesise progesterone from pregnenolone. The FSH and LH receptors are 7-transmembrane G-protein coupled receptors which share similar post-receptor mechanisms, activating cyclic AMP and inducing genes downstream of PKA (Simoni et al., 1997), p38 MAPK (Gonzalez-Robayna et al., 2000) and AP-1 (Sharma and Richards, 2000) pathways.

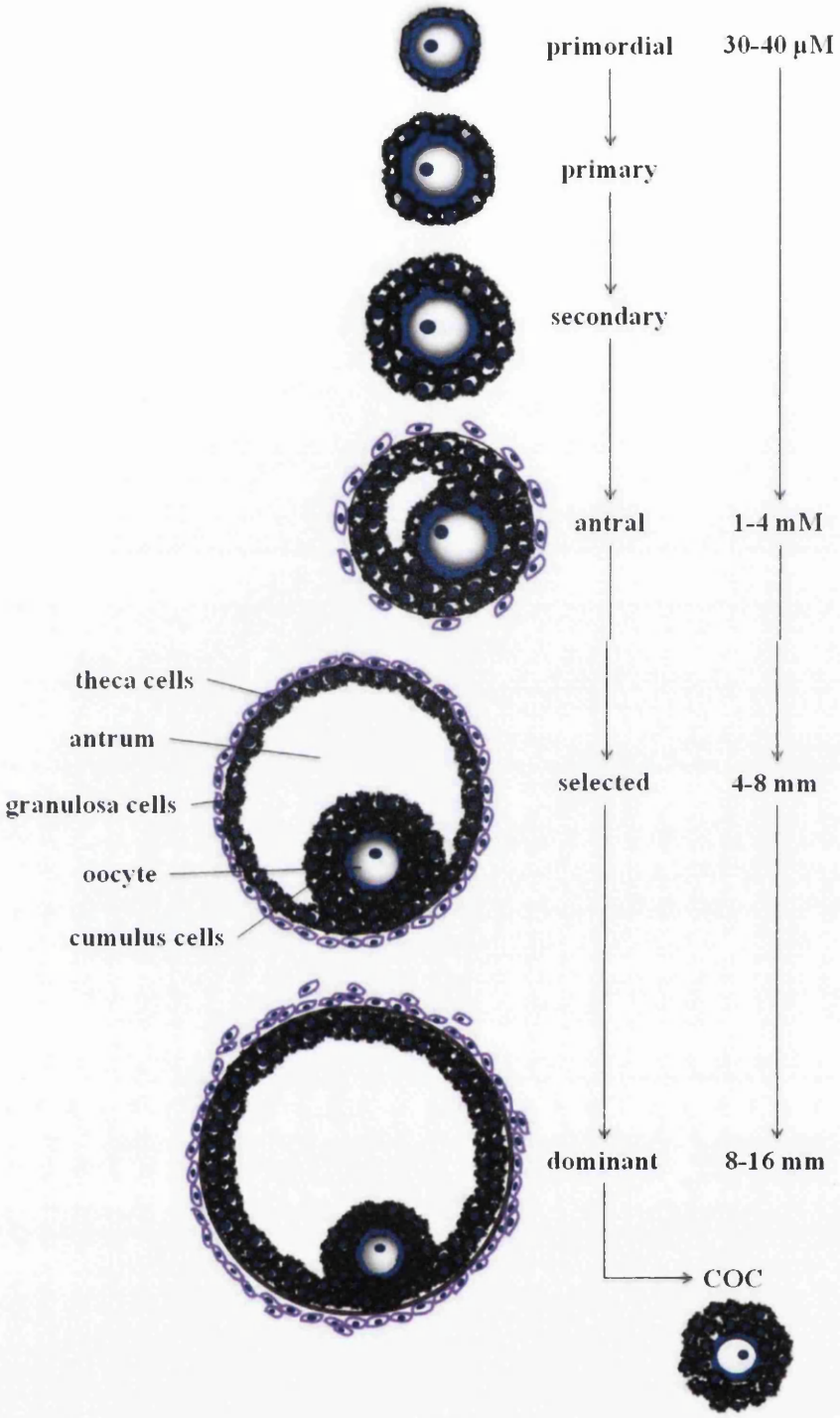


Figure 1.4 Follicles grow from 30 µM to around 16 mm over a period of 180 days. Cows are born with thousands of primordial follicles, which develop into primary and secondary follicles. Antral follicles are characterised by the formation of a fluid filled space (or antrum). Each wave, 6-24 follicles emerge as selected and usually one will become dominant, potentially ovulating a cumulus-oocyte complex.

1.6 Follicle waves

In cattle, follicles develop in follicular waves, with most oestrous cycles consisting of 2 or 3 waves, each lasting 6 to 9 days (Evans et al., 1994) (Fig 1.5). A cohort of 6 to 24 ovarian follicles synchronously emerge in response to increased concentrations of follicle stimulating hormone in peripheral plasma (Fortune et al., 2001). The concentration of FSH begins to decline after a further 2 days and only larger follicles with more FSH receptors are able to survive, whilst remaining follicles undergo atresia. As FSH concentrations continue to fall, one follicle is selected as the dominant follicle, becoming responsive to luteinising hormone. In cows actively immunised against GnRH, FSH treatment alone only stimulates development of a low number of medium-sized follicles which are oestradiol inactive, and no follicles reach dominance (Crowe et al., 2001). Interestingly, any viable follicle is capable of becoming dominant, as cows can be super-ovulated with FSH (Adams et al., 1993, Ireland et al., 2007); one randomly selected 5 mm follicle can be directed to become dominant if all other follicles of a similar size are destroyed (Gibbons et al., 1997), and the subordinate follicle will become dominant if the dominant follicle is ablated prior to day 15 of the oestrous cycle (Dean and Dailey, 2011). However, it seems the follicle selected is not random, rather that the follicle has a developmental advantage over the other follicles, such as being closer to the ovarian vasculature (Fraser, 2006, Acosta, 2007).

The future dominant follicle emerges 7 h earlier than the largest subordinate follicle (Kulick et al., 1999). Prior to deviation, all follicles grow at the same rate (Ginther et al., 1997). However, because the future dominant follicle emerges first, it is larger and thus maintains its advantage over the other follicles in the cohort. Thus, the larger follicle increases its surface area at a greater rate, reflected by more GCs within the follicle and more FSH receptors. As FSH concentrations decline, the larger follicle is still able to grow, not only due to its greater number of FSH receptors, but also the presence of LH receptors on GCs within dominant follicles. Activation of the LH receptor leads to production of androstenedione, which GCs convert to oestradiol. Higher concentrations of oestradiol within the follicle increase granulosa responsiveness to FSH, further protecting the dominant follicle from atresia. The greater response to FSH in the larger follicle also increases the production of insulin-like growth factor (IGF) binding protein proteases (Rivera and

Fortune, 2001), which increase the concentration of bio-available IGF-1 within the follicle (Rivera and Fortune, 2003). The bio-available IGF-1 acts synergistically with FSH to increase the production of oestradiol by the dominant follicle (Adashi, 1998), which then suppresses FSH thus starving the smaller follicles of their gonadotrophin drive (Burke et al., 1996, Ginther et al., 2000). It has also been hypothesised that other substances produced by the dominant follicle may suppress FSH including inhibin (Lussier et al., 1993, Kaneko et al., 1997), which may be synthesised by the dominant follicle in response to falling concentrations of FSH, thus further suppressing the subordinate follicles (Mihm et al., 1997).

The fate of the dominant follicle depends on whether the corpus luteum from the previous oestrus cycle has luteolysed. When a corpus luteum is present, the circulating concentration of progesterone is high and LH pulsatile frequency is low. In this situation, the dominant follicle undergoes atresia, FSH is no longer suppressed and a new wave of follicle growth begins (Roche, 1996). If luteolysis has occurred, LH pulse frequency increases, circulating concentrations of progesterone are low and the dominant follicle persists. The stimulatory effect of LH on GCs also induces expression of epidermal growth factor (EGF)-like factors in GCs that propagate the LH signals through the follicle to the cumulus cells and oocyte (Portela et al., 2011), which do not express the LH-receptor (Peng et al., 1991, Park et al., 2004, Ashkenazi et al., 2005, Jamnongjit et al., 2005). These factors cause resumption of meiosis in the oocyte and formation of a hyaluron-rich extracellular matrix (Lorenzo et al., 1994, Lonergan et al., 1996, Hosoda and Terada, 2007). This matrix leads to cumulus expansion, which causes a concurrent increase in antrum volume, forming a blister on the follicle (Fulop et al., 2003). Cyclic AMP stimulates plasminogen activator, which catalyses the breakdown of plasminogen to plasmin (LeMaire, 1989). Activation of LH also increases latent collagenase, which seems to be activated by plasmin (Woessner Jr et al., 1989). Active collagenase digests the follicular tissue at the blister site creating a hole, known as a stigma, through which the cumulus-oocyte complex exits. The retained mural GCs within the follicle become luteinised, producing progesterone and the remaining follicle forms a highly vascularised corpus luteum. The oocyte is wafted by cilia towards the fallopian tube, at which point, the oocyte has finished meiosis I, yielding a large secondary oocyte which contains all cytoplasmic material, and a smaller polar body.

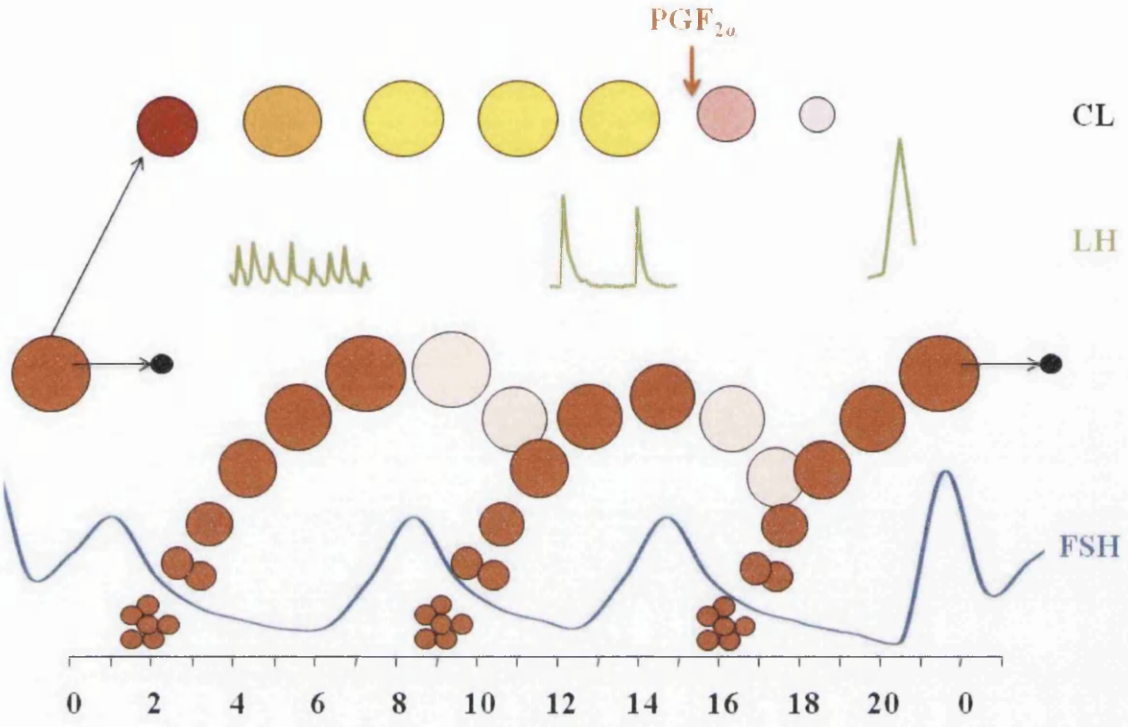


Figure 1.5 Bovine ovarian follicles develop in waves (3-wave model). After ovulation, an increase in FSH (shown in blue) triggers recruitment of a cohort of small antral follicles (orange circles). Most of these undergo atresia until there is one dominant follicle remaining. The previously ovulated follicle forms a corpus luteum (CL), releasing progesterone which prevents the necessary rises in LH (green) for ovulation, thus the dominant follicle undergoes atresia (cream circles) and a second rise in FSH triggers a new wave of follicles to emerge. Concentrations of progesterone remain high through this second wave of growth thus all follicles undergo atresia, triggering a third rise in FSH and a new wave of follicular growth. When a cow is not pregnant, the uterus releases $PGF_{2\alpha}$, causing luteolysis and thus progesterone concentrations fall. This enables a sufficient peak in LH to trigger ovulation of the dominant follicle.

1.7 Oocyte maturation

The oocyte is the female gamete, containing a rich cytoplasm and a nucleus. During early embryo development, primordial germ cells that develop into oogonia are surrounded by somatic cells and form primordial follicles. The oogonia enter meiosis I during foetal development, arresting at the end of prophase I. The egg produces cAMP, which inactivates cyclin B, maintaining the meiotic arrest until the pre-ovulatory surge in LH triggers an increase in maturation promoting factor (MPF) (Aktas et al., 1995). Oocyte maturation, when MPF releases the oocyte from meiotic release, consists of nuclear and cytoplasmic maturation and begins at the end of prophase I, continuing until the egg is once again arrested at metaphase II. During nuclear maturation, the nuclear membrane begins to fold, the nuclear pores disappear and the nuclear membrane fragments. These events are collectively termed as germinal vesicle breakdown (GVBD). Following GVBD, the chromosomes condense, separate, migrate to the poles (anaphase I), become surrounded by a nuclear membrane (telophase I) and the first polar body is extruded. Cytoplasmic maturation refers to the redistribution of cytoplasmic organelles, reorganisation of the cytoskeleton and storage of oocyte-derived mRNAs, which will support fertilisation and early embryo development of the fertilised mature oocyte (Ferreira et al., 2009).

At the end of anaphase I, levels of MPF rapidly drop and the oocyte is once again arrested at metaphase II, during which a second meiotic division takes place without chromosome replication (Wu et al., 1997). This decrease in chromosome number is essential to ensure that the fertilised embryo is restored to a diploid cell. At fertilisation, binding of the sperm to the egg triggers a rise in the intra-oocyte calcium concentration (Fissore et al., 1995, Ross et al., 2008). This leads to egg activation, when an oocyte becomes competent to begin embryo development. Oocyte activation is characterised by resumption of meiosis II, cortical granule exocytosis to prevent polyspermy and extrusion of the second polar body (Liu et al., 2009).

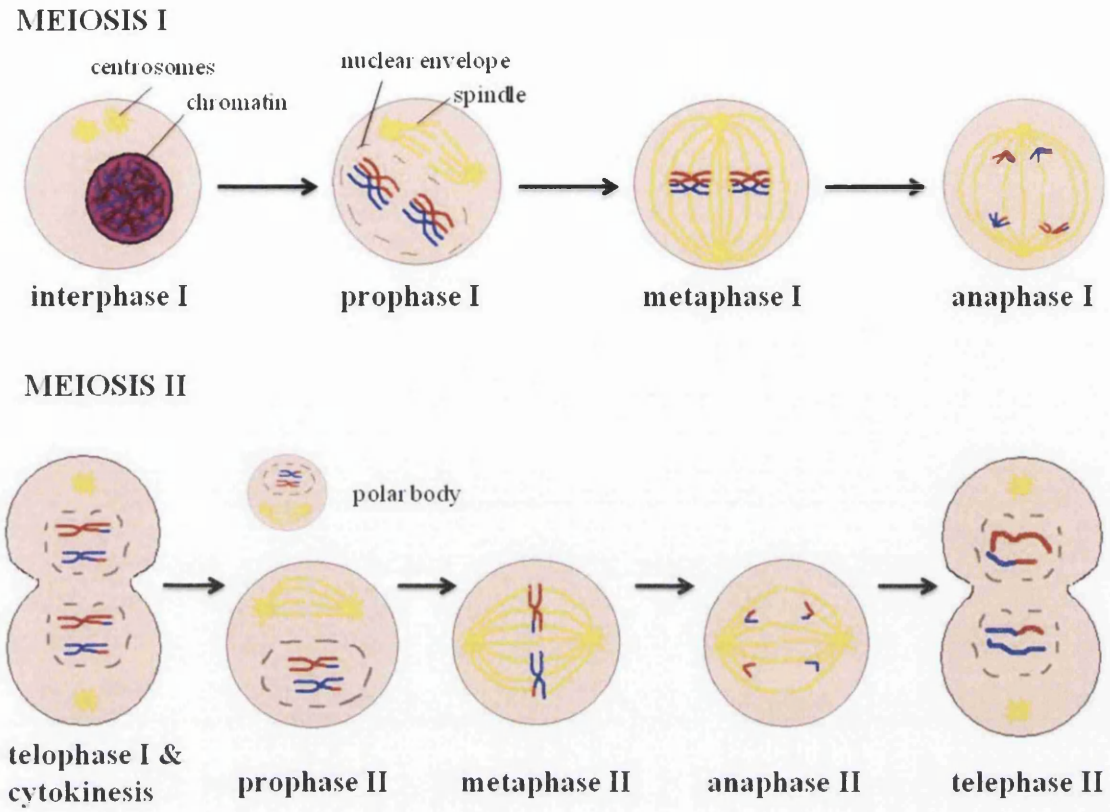


Figure 1.6 The oocyte divides by meiosis. Prior to birth, the oocytes grow through interphase I, when chromosomes duplicate, and arrest at the end of prophase I, when the nuclear membrane has begun to break down and homologous recombination takes place. In response to the preovulatory surge in LH, the oocyte continues to mature, with the nuclear membrane breaking down during prophase I, microtubule spindles attaching to the chromosomes (metaphase I) and homologous chromosomes being separated (anaphase I). During telophase I, the nuclear membrane reforms around pairs of chromosomes and a secondary oocyte and a polar body are formed through cytokinesis. The polar body disintegrates and the secondary oocyte arrests at metaphase II until fertilisation, when sister chromatids separate, the oocyte matures and a second polar body is formed.

1.8 Communication between cumulus cells and the oocyte

At the start of follicular development, GCs form contact sites with the oolemma, consisting of cytoplasmic projections extending from a single granulosa cell, which traverse the zona pellucida and form gap junctions at the oolemma. Called transzonal projections (TZPs), these specialised membrane structures, which connect the cytoplasm of adjacent GCs, are formed at the primordial stage of follicle development by the docking of two hemi-channels around an aqueous pore to form intercellular channels. These allow the transfer of small metabolites, nucleotides and second messengers up to 1 kDa in size between cells (Gershon et al., 2008). It has also been postulated that gap junctions are involved in cytoplasmic maturation of the oocyte (Eppig et al., 1996) and in maintaining meiotic arrest of oocytes. Transfer of inhibitory cAMP from somatic cells to the oocyte maintains meiotic arrest, with reinitiation of meiosis occurring if communication between cells is disrupted, as occurs during the pre-ovulatory surge in LH (Larsen et al., 1981).

Transzonal projections are formed from cytoskeletal components, including F-actin and microtubules, which provide tracks for translocation of organelles of the secretory pathway, such as lysosomes (Albertini et al., 2001). Use of Mitotracker, a fluorescent dye which stains mitochondria, has demonstrated the bidirectional movement of mitochondria and translocation of endosomes and lysosomes across the zona pellucida (Albertini et al., 2001). The TZPs have been shown to deliver factors derived from the follicle cells, such as leptin and STAT3 to the oocyte (Antczak and Van Blerkom, 1997), which may influence oocyte metabolism. Albertini *et al* (2001) proposed a model for delivery of paracrine factors to and from the oocyte, which is summarised in Fig 1.7. During *in vitro* bovine oocyte maturation, the density of TZPs increases in response to hormonal changes in the media (Allworth and Albertini, 1993). During periods of oocyte growth, TZPs extend as deep invaginations, invading the germinal vesicle. Preantral follicles contain the most TZPs with retraction of TZPs during antrum formation, decreasing the number of connections between oocyte and somatic cells (Makabe et al., 2006). GDF-9 has been shown to be an important protein in the development of TZPs; healthy mice develop TZPs anchored by F-actin but GDF-$9^{-/-}$ knockout mice develop TZPs that are not anchored but envelop the surface of the oocyte (Carabatsos et al., 1998).

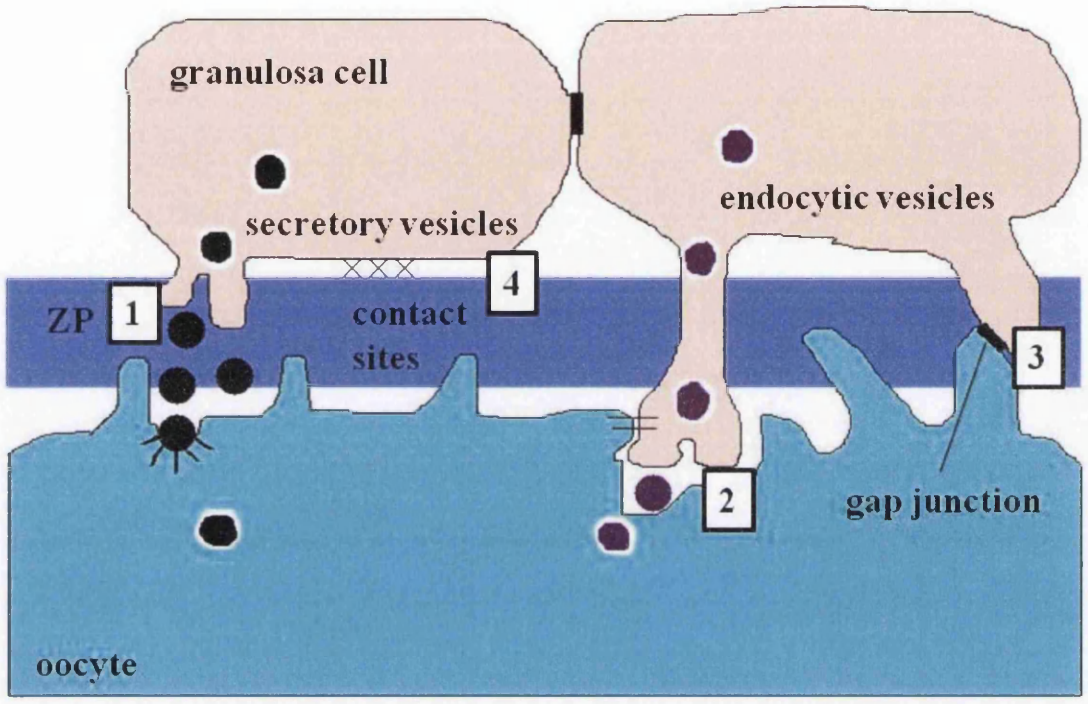


Figure 1.7 A potential model for the delivery of paracrine factors between GCs and the oocyte. (1) Secretory vesicles transport granulosa cell-derived factors to the oocyte, where they are endocytosed. (2) Oocyte factors are endocytosed at attached sites of TZPs to the oolemma. Endocytic vesicles traffic along microtubules to the body of the granulosa cell, where intracellular processing may take place. (3) Gap junctions allow for direct communication between granulosa cell and oocyte or between GCs. (4) Anchoring between the granulosa cell and the zona pellucida (ZP) is necessary for correct orientation of TZPs. Black circles represent granulosa cell-derived factors and purple circles represent oocyte-derived factors. Figure adapted from (Albertini et al., 2001).

1.9 Early embryogenesis

Meiosis II starts straight after meiosis I has finished, but the oocyte is arrested at metaphase II until fertilisation, when the cell membrane of the oocyte and sperm fuse. Then, the oocyte resumes its second round of meiosis, producing a haploid ovum and releasing a second polar body. The sperm forms a pronucleus and its tail and mitochondria degrade, before fusing with the ovum (Okamura and Nishiyama, 1978). The first stage to occur after fertilisation is holoblastic cleavage, where mitotic divisions occur every twelve to twenty-four hours without the zygote growing, resulting in blastomere cells (Gilbert, 2000). The first division occurs across the meridional axis of the egg with second division perpendicular to this, thus producing four cells (Gilbert, 2000). Rotational cleavage then takes place until midblastular transition when maternal-zygotic transition occurs with degradation of maternal RNAs and synthesis of embryonic RNAs and embryonic transcription begins (Schier, 2007). Prior to the initiation of embryonic transcription, maternal effect genes play a key role. The products of these genes from the maternal genome are required for key steps in embryonic development, such as establishment of meiosis and determination of the body patterning (Tadros and Lipshitz, 2009). In the cow, maternal effect genes include *ZAR1, GDF9* and *NLRP5* (also known as *MATER*). These genes are expressed by bovine oocytes and zygotes, up until the 8 cell stage (Pennetier et al., 2004). Although the role of these genes is yet to be fully elucidated, especially in domestic animals, fertilised oocytes from *Nlrp5*$^{-/-}$ knockout mice fail to progress beyond the 2 cell stage (Tong et al., 2000) and embryos from *Zar1*$^{-/-}$ knockout mice mostly arrest prior to cleavage (Wu et al., 2003).

When the zygote reaches eight blastomeres, the cells compact, become polarised and form tight junctions between cells on the outside of the cellular ball. This leads to the development of two populations: trophoblast cells on the outside and apolar blastomeres which form the inner cell mass. The trophoblast cells act as a sodium pump, and later produce the chorion, the embryonic part of the placenta (Gilbert, 2000). The water which enters the cell through osmosis forms a blastocoel cavity thus the zygote is now known as an early blastocyst. The inner cell mass is pushed to one side of the blastocoel cavity and later gives rise to the embryo and some extraembryonic membranes.

It is essential for the development of the embryo and uterus to synchronise for successful implantation to occur (Rowson et al., 1972). In the cow, attachment to the uterus occurs between 30 and 55 days after ovulation with chorionic villi making contacts with maternal tissues in the crypts of caruncles, eventually forming the functional unit of the placentome (Brinster, 1974). This protracted pre-implantation period allows the bovine blastocyst to hatch from inside the zona pellucida and change in shape, becoming first tubular and then filamentous, filling the entire uterine horn (Bazer et al., 2009). This filamentous blastocyst consists mainly of extra-embryonic trophectoderm lined with entoderm (Spencer et al., 2008). Progesterone stimulates blastocyst growth and when elongated, the trophoblast produces interferon τ, which acts on the endometrium to inhibit release of prostaglandin $F_{2\alpha}$ ensuring continued production of progesterone by the corpus luteum (Bazer et al., 1991).

1.10 Uterine disease

Uterine disease, pelvic inflammatory disease (PID), endometritis and metritis are generic terms used to describe the disease following protozoal, fungal, viral or bacterial infection of the upper female genital tract and are most common in humans and cattle. Infection leads to activation of the innate immune system and inflammation. Uterine disease can cause infertility through disruption of endocrine function in the reproductive tract, direct damage caused to the reproductive organs and through the effect of infection on ovarian and uterine function.

In humans, most cases of PID are associated with sexually transmitted infections, for example chlamydia, caused by the obligate intracellular gram negative bacterium *Chlamydia trachomatis.* Up to forty per cent of untreated chlamydia cases will develop symptomatic PID with post-infection damage to fallopian tubes causing a third of all cases of female infertility (W.H.O, 2011). In the United States of America, it is estimated that over one million women seek treatment for PID every year. However, as uterine infection can be symptomless, the true number of people affected could be greater. Each year, one hundred thousand American women become infertile and one hundred and fifty die from PID or further complications (N.I.A.I.D, 2009).

Bacterial contamination of the bovine uterus is ubiquitous after parturition with 40% of cows developing clinical disease within 10 days of calving (metritis), 20% suffering from a prolonged clinical disease for at least 3 weeks post-partum (endometritis) and 30% having subclinical endometritis (Sheldon et al., 2009). These infections impact on fertility and milk yield, as well as causing pain and suffering to the animal. The economic cost of this is estimated at €290 per case when reduced milk production, delayed conception and treatment costs are taken into account (Drillich et al., 2001). This results in an estimated annual cost of around €1.4 billion for the European Union alone (Sheldon et al., 2009). Post partum infection leads to activation of the innate immune system, influx of neutrophils into the endometrium and inflammation. In subclinical endometritis, inflammation of the endometrium leads to a significant decrease in reproductive performance, but occurs without signs of clinical endometritis (Sheldon et al., 2009). Subclinical endometritis is defined by the presence of neutrophils exceeding five-and-a-half per cent of all cells in uterine flushes taken more than five weeks post partum and affects up to three quarters of all dairy cows. Animals with subclinical disease take longer to conceive and have reduced conception rates (Salasel et al., 2010, Moges and Jebar, 2012). There are three grades of clinical metritis: animals with grade one metritis have an enlarged uterus and purulent discharge; grade two cows have symptoms as stage one but also with signs of a systemic illness, for example, hyperthermia, and grade three cattle have signs of toxaemia, such as decreased appetite (Sheldon et al., 2009). The grading of metritis is directly related to prognosis.

During pregnancy, the bovine uterus is sterile, but contamination of the post-partum uterus is ubiquitous. The most prevalent bacteria isolated from the postpartum bovine uterus are *Escherichia coli* and *Truepurella pyogenes* (Sheldon et al., 2002). A number of anaerobic bacteria are also present, although other bacteria can be isolated from the uterus of cows with or without uterine disease. *Escherichia coli* appears to prime the uterus for infection by bacteria, such as *T. pyogenes*, thus both Gram negative and Gram positive bacteria are isolated from the uteri of animals with metritis (Sheldon et al., 2009). Bacteria are expelled through uterine contraction and regeneration of the endometrium; often the host immune system is able to overcome any infection and clinical disease may not occur. Risk factors for developing uterine disease include placental retention and dystocia (Dubuc et al.,

2010). Birth of twins is also a risk factor because it causes stretching of the uterus, resulting in ineffectual uterine contraction, increased risk of retained foetal membranes and reduced expulsion of contaminating bacteria (Potter et al., 2010).

Most cows will clear bacterial contamination within three weeks of parturition without need for treatment. However, treatment is needed for clinical endometritis after three weeks post partum, as the chance of the cow spontaneously clearing the infection within a 2 week period is only thirty-three per cent, compared to double that for pharmaceutical treatment (Sheldon, 2007). Oestrus is one of the most effective ways of clearing uterine infection, achieved by administering intramuscular $PGF_{2\alpha}$, which is luteolytic, thus decreasing progesterone if a corpus luteum is present. Progesterone suppresses uterine immune defences by decreasing lymphocyte proliferation: ovariectomised ewes show increased lymphocyte proliferation and exogenous progesterone causes decreased lymphocyte proliferation (Lewis, 2004). Thus by decreasing production of progesterone, the immune response of the cow can be enhanced. Administration of prostaglandins also causes release of leukotriene B4, which promotes uterine involution, thus enhancing clearance of uterine infection and contamination (Lewis, 2004). In cows without a functional corpus luteum, exogenous PGF_{2a} may enhance uterine involution and contractility (Hirsbrunner et al., 2003). An alternative treatment for endometritis is intra-uterine infusion of antimicrobials, such as cephalosporin, with non-steroidal anti-inflammatory co-therapy when systemic signs are present (Drillich et al., 2007).

Escherichia coli is a gram negative, anaerobic bacterium and part of the normal flora of the gastrointestinal tract. The bacterium is rod-shaped and up to 2 μm long, with some strains using flagella for motion. The lipopolysaccharide (LPS) outer layer consists of lipid A, which is responsible for the *E. coli's* toxicity, and an O-antigen, which is highly variable and consists of repeated oligosaccharide units (Beutin et al., 2007). A protective capsule surrounds the bacterium, which consists of K-antigen proteins (Whitfield and Roberts, 1999). Until recently, it was presumed that the bacteria that colonise the postpartum uterus are a random collection of *E. coli* strains from the gastrointestinal tract, urinary tract and environment. Interestingly, it now seems that in bovine uterine disease, specific strains of *E. coli* are pathogenic in the endometrium, especially strains in phylogenetic groups A and B1 (Sheldon et al., 2010).

Uterine infections not only impact on endometrial health, but also on the function of the hypothalamus, pituitary and ovary. Although uterine disease does not perturb the secretion of FSH by the pituitary, intra-uterine LPS infusion prevents the pre-ovulatory surge of LH, thus preventing ovulation (Peter et al., 1989). This perturbation of LH signalling has been observed in other species, including pigs (Jana et al., 2004) and sheep (Battaglia et al., 1999), in which peripheral infusion of LPS suppresses pulsatile LH secretion from the pituitary and GnRH secretion from the hypothalamus. Interestingly, LPS infusion blocks the pre-ovulatory rise in oestradiol concentrations even in animals with normal LH pulsatility, suggesting direct effects of LPS on the ovary (Battaglia et al., 2000). Animals with metritis have an increased risk of a prolonged luteal phase and cows with abnormal vaginal discharge are more likely to have delayed ovulation, decreasing fertility (Opsomer et al., 2000). Post-partum cows with a high bacteria growth from transcervical swabs had a smaller first dominant follicle and lower plasma oestradiol concentrations than those with low bacterial growth (Sheldon et al., 2002) and also had lower serum progesterone concentration, a smaller corpus luteum, an increased risk of a persistent dominant follicle and a decreased chance of ovulation (Williams et al., 2007). Interestingly, it seems that *E. coli* is the main organism to cause ovarian dysfunction as uterine infusion of *T. pyo* had no effect on FSH, LH, oestradiol or progesterone concentrations, nor did this effect follicle wave emergence, dominant follicle diameter or corpus luteum diameter (Miller et al., 2007), whereas LPS treated animals were less likely to ovulate (Williams et al., 2008). Although many of these studies have examined the effects of clinical endometritis on ovarian health, a recent study found that animals with subclinical endometritis were less likely to have a corpus luteum (19% vs 55%) and fewer had returned to cyclicity after parturition than healthy cows (Green et al., 2011). These animals with subclinical endometritis also had altered hormone concentrations in follicular fluid, with increased cortisol and decreased testosterone, oestradiol, androstenedione and DHEA.

In a long-term study in Germany, infertility was the reason for almost a third of all bovine culling (Frerking, 1999). This high incidence of culling due to infertility, a major cause of which is uterine disease, leads to farms having to maintain a larger herd size to allow for the wastage within the system. This has an environmental impact, increasing the greenhouse effect and using valuable land.

Although livestock farming could be argued to be highly energy inefficient, there is little chance of consumer appetite for meat and dairy products reducing, thus methods to minimise the environmental impact are likely to become more important as the global population and wealth increases.

Escherichia coli paves the way for further infection by other bacteria and LPS is the part of *E. coli* recognised by the immune system, triggering a response characterised by release of pro-inflammatory cytokines (Dohmen et al., 2000). Thus, *in vitro* experiments focussed on the response of uterine cells to LPS, finding that endometrial cells express the molecular machinery necessary to detect LPS (Herath et al., 2006) and that LPS treatment switches endometrial prostaglandin production to favour PGE, thus providing a mechanism to explain the prolonged luteal phase often observed *in vivo* (Herath et al., 2009a). *In vivo,* endometrial expression of pro-inflammatory cytokines is higher in cows with uterine disease (Chapwanya et al., 2009, Herath et al., 2009b), and *in vitro,* endometrial cells increase expression of mRNA and protein accumulation of pro-inflammatory cytokines in response to LPS (Cronin et al., 2012). An interesting and relevant paper found that LPS collects in the ovarian follicular fluid of infected cows, with the concentration of LPS correlating to the clinical diagnosis (highest in cows with endometritis, lower in cows with subclinical endometritis, undetectable in healthy cows) and, although the concentrations found in follicular fluid are lower than those found in the uterus (Williams et al., 2007), the concentration is higher than found in the plasma of diseased animals (Dohmen et al., 2000), thus the GCs of infected cows are bathed in LPS. It was further found that *in vitro* treatment of GCs with LPS perturbs their oestradiol production and that GCs express the receptor necessary to detect LPS (Herath et al., 2007). Thus, the question arose of whether ovarian cells have an innate immune function.

1.11 Mastitis

In the previous 50 years, dairy conception rates have fallen from around 65% to 38%, with a concomitant increase in milk yield (Crowe and Williams, 2012). Associated with this increased milk yield is mastitis (infection of the mammary gland), which is mainly caused by bacteria. Acute bovine mastitis is caused by Gram negative organisms (mainly *E. coli*) and is associated with clinical signs, such as swollen, hard or painful udders. Subclinical bovine mastitis is caused by Gram positive organisms, such as *Staphylococcus aureus*, has no clinical signs and can last for several months (Huszenicza et al., 2004, Lavon et al., 2010). Both forms of mastitis decrease milk production and increase the concentration of cytokines in milk and the circulation (Hansen et al., 2004). In addition to perturbing mammary function, around 30% of infected animals also experience endocrine disruption and abnormal ovarian steroidogenesis. Animals with subclinical mastitis produce less oestradiol and androstenedione and have lower expression of mRNA encoding key enzymes (including $P450_{scc}$, $P450_{17OH}$ and $P450_{arom}$) for a prolonged period, whereas animals with clinical mastitis have lower production of oestradiol and androstenedione only during the first oestrous cycle (Lavon et al., 2011a, Lavon et al., 2011b). In addition, animals with moderate to severe mastitis have fewer secondary and dominant follicles (Rahman et al., 2012). An experimental model of acute mastitis, induced through intra-mammary injection of LPS, found that animals with induced disease produced less oestradiol, had delayed ovulation and a suppressed or delayed LH surge compared to healthy controls (Lavon et al., 2008). It is currently unknown whether the ovarian follicular fluid of animals with mastitis contains LPS or other pathogen-associated molecular patterns (PAMPs), as occurs in animals with uterine disease. The perturbation of ovarian function could thus be direct, or through indirect causes, such as the increase in circulating cytokines. What is clear, however, is that infections distant to the ovary perturb ovarian function.

1.12 Immunity

The immune system is an organism's defence against infection and disease, detecting and destroying pathogens, such as bacteria and viruses. Infection is the colonisation of a host by a foreign pathogenic species. The immune system consists of a number of layers of developing complexity and specificity. Most simply, skin acts as a physical barrier to prevent bacteria from entering the body. Once a foreign object enters the body it encounters first the innate immune system and then the adaptive immune system. The innate immune system is present in all plants and animals, but only jawed mammals have developed adaptive immunity (Matsunaga and Rahman, 1998). The success of the immune system depends on the ability to distinguish self from non-self, through recognition of PAMPs. The immune system also needs to identify self-molecules expressed by damaged tissues, such as damage-associated molecular patterns (DAMPs).

When a bacterium enters the body, PAMPs activate Toll-like receptors. Most PAMPs are conserved structures which can be distinguished by the immune system as non-self and are common to many related pathogens (Zhong and Kyriakis, 2007). Activation of a TLR triggers an intracellular signalling cascade, resulting in expression of inflammatory and immune mediators including cytokines, chemokines and prostaglandins (Takeda and Akira, 2005, Kim et al., 2011). Cytokines are small peptides below 30kDa involved in many processes including proliferation, differentiation, death and survival. These cytokines aid recruitment of immune cells to the area of infection and cause inflammation. For the present project, the innate immune system and the inflammatory response it elicits in the body was of most interest, thus the adaptive immune system will only be briefly described in Table 1.1 below. Prior to ovulation, ovarian follicles do not contain professional immune cells or antigen-presenting cells (Spanel-Borowski et al., 1997, Bromfield and Sheldon, 2011). However, as described above in 1.10, a recent study has described the presence of innate immune receptors on GCs and the perturbation of granulosa cell function in response to the prototypical PAMP, LPS (Herath et al., 2007). Thus, this review will focus on the function of these innate immune receptors, called Toll-like receptors.

Table 1.1 Differences between the innate and adaptive immune responses. The innate immune response is a non-specific response with receptors that have broad specificity. The adaptive immune response occurs later and is specific. For this project, the innate immune response is of interest, because ovarian follicles are devoid of antibody-producing cells prior to ovulation.

Innate Immune Response	**Adaptive Immune Response**
Pathogen is recognised by receptors generated in germline cells	Pathogen is recognised by receptors generated through spontaneous mutation
Receptors have broad specificity, i.e. recognise many related molecular structures 'PAMPs'	Receptors have narrow specificity, they recognise a particular epitope
Receptors are pattern recognition receptors	Receptors are B-cell and T-cell receptors for antigen
PAMPs are polysaccharides and polynucleotides that differ little from one pathogen to another but are not found in the host	Epitopes are derived from polypeptides and reflect the individuality of the pathogen
Response is immediate, but with no memory of previous exposure	Response takes 3-5 days to develop clones of responding cells, but retains memory of previous exposure
Occurs in all metazoans	Occurs in jawed vertebrates only

1.12.1 Toll-like Receptors

Toll-like receptors (TLRs) are membrane bound proteins involved in recognising pathogens and are expressed by numerous cell types including professional immune cells (Kawai and Akira, 2011), endometrial cells (Davies et al., 2008), ovarian GCs (Herath et al., 2007) and cumulus-oocyte complexes (Shimada et al., 2006). They act as pattern recognition receptors (PRRs) to bind molecules associated with bacteria, viruses and fungi (PAMPs) or endogenous ligands released during cell necrosis (DAMPs). Toll was originally discovered in Drosophila as an essential receptor for the establishment of the dorsal-ventral pattern in developing embryos (Anderson et al., 1985). Flies with mutant Toll showed increased susceptibility to fungal infections, which suggested a roll for Toll in immunity, as well as providing an interesting link between two ancient mammalian systems: immunity and reproduction (Lemaitre et al., 1996). A mammalian homologue of the Drosophila Toll receptor was later shown to induce expression of genes involved in inflammation and immunity (Medzhitov et al., 1997). Toll-like receptors are type I integral membrane glycoproteins with a horseshoe shaped structure, characterised by an extracellular leucine rich domain containing 19-25 tandem leucine rich repeats and a cytoplasmic signalling domain, homologous to that of the interleukin-one receptor (TIR domain) (Akira et al., 2006). Each leucine (L) rich repeat has the structure XLXXLXLXX (X being hydrophilic residues) and consists of an alpha-helix and a beta-strand connected by an asparagine ladder (Werling et al., 2009).

To date, eleven different TLRs have been identified in humans (Zhang et al., 2004), ten TLRs in cow (Menzies and Ingham, 2006, Davies et al., 2008) and 13 in mice (Wu et al., 2008). Each TLR binds different PAMPs (Table 1.2) and sensing of the PAMP can occur on the cell surface or within endosomes or lysosomes (Akira et al., 2006). The location of the TLR is important for correct PAMP recognition and to prevent incorrect sensing of self. Toll-like receptor 1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are found intracellularly (Barreiro et al., 2009). Of particular interest to this thesis are TLR1, TLR2, TLR4, TLR5 and TLR6, as these are extracellular receptors that sense bacteria. Studying the recognition of Gram negative and Gram positive bacteria is important for these studies, as both types of bacteria are associated with

endometritis and mastitis. These infections are caused by the Gram negative bacterium *E. coli,* whose LPS binds TLR4 and flagellin (part of flagella used by some strains for motility) binds TLR5, and by Gram positive bacteria, such as *S. aureus*, whose lipoproteins are recognised by TLR2 (Bubeck Wardenburg et al., 2006, Volz et al., 2010, Gilbert et al., 2013). Ultra-purified forms of LPS and flagellin will be used in experiments. However, the choice of ligand for TLR2, which forms heterodimers with TLR1 and TLR6 to recognise triacylated and diacylated lipoproteins, respectively (Schenk et al., 2009), is not so clear cut. Although peptidoglycan, a major component of Gram positive bacterial cell walls, was considered to be a specific TLR2 agonist, it has recently been shown to activate the intracellular pathogen-sensing NOD-like receptors (Girardin et al., 2003, Sorbara and Philpott, 2011, Mo et al., 2012). Other TLR2 ligands include lipoteichoic acid (LTA) and lipoglycans, which bind TLR2, but seem to also require other TLRs for maximal response. Treatment of TLR4 deficient murine macrophages with LTA suppressed the response to this PAMP, suggesting that LTA is either also recognised by TLR4, or that other contaminating PAMPs, such as LPS, may not have been removed during purification (Takeuchi et al., 1999), a recognised problem in preparations of endogenous ligands (Tsan and Gao, 2007). Therefore, in order to investigate the responses of granulosa cells and oocytes to TLR2 ligands, Pam3CSK4, a synthetic triacylated lipoprotein, will be used to ensure greater target specificity.

Damage-associated molecular patterns are endogenous molecules released by cells after necrosis and are also TLR ligands. A number of DAMPs have been described as causing cytokine release through activation of Toll-like receptors, including high mobility group box one (HMGB1) and heat shock proteins (HSPs) (Andersson et al., 2000, Yang et al., 2005). However, the effects seen in response to HMGB1 and HSPs may be due to impurities in the preparations, derived from bacterial sources (Tsan and Gao, 2009, Tsan, 2011), thus whether any endogenous ligands are true TLR ligands remains controversial. Hyaluronic acid (HA) is a glycosaminoglycan, which, in mammals, is synthesised by one of three synthases. This produces different length polymers, with high-molecular weight (HMW) HA (over 500 kDa) tending to have anti-inflammatory actions and lower molecular weight HA being pro-inflammatory.

Table 1.2 Common pathogen-associated and damage-associated molecular patterns that activate specific Toll-like receptors.

Toll-like Receptor	PAMP ligand	DAMP ligand
1	Bacterial lipopeptides Soluble lipoproteins	
2	Lipoteichoic acid (LTA) Bacterial lipoproteins and glycolipids Fungal zymosan Porins	Heat shock proteins HMGB1
3	Double-stranded RNA tRNA (fungal)	Polyinosine-polycytidylic acid mRNA tRNA (host)
4	Lipopolysaccharide (LPS)	Fibrinogen Hyaluronic acid fragments Heat shock proteins Defensins HMGB1
5	Flagellin	
6	Mycoplasma lipopeptides	
7	Viruses (single stranded RNA)	
8	Viruses (single stranded RNA)	
9	Unmethylated CpG motifs in bacterial DNA	DNA HMGB1
10	Unknown	

In this project, HMW HA and ultra-low molecular weight HA (ULMW HA) will be used, with molecular weights of over 950 kDa and under 8 kDa, respectively. Low-molecular weight hyaluronic acid has been shown to increase production of pro-inflammatory cytokines by chondrocytes (Campo et al., 2010), activate TLR4 on dendritic cells (Termeer et al., 2002) and bind TLR2 and TLR4 on murine cumulus cells (Shimada et al., 2006). This is of interest because hyaluronan is produced by cumulus cells within the ovary prior to ovulation (Assidi et al., 2010). During murine pregnancy, HA increases to 71% of total cervical glycoaminoglycan and the activity of hyaluronidase (the enzyme which breaks down HA) increases during labour (Akgul et al., 2012). Thus, post-partum, a large amount of low-molecular weight, pro-inflammatory HA may be able to reach the ovary, where, potentially, it could act as a DAMP.

Binding of an appropriate ligand to a TLR activates an intracellular signalling cascade. The two main pathways are the MyD88-dependent (myeloid differentiation primary response gene 88) and the MyD88-independent pathways. The MyD88-dependent pathway is used for all TLRs except TLR3 and the MyD88-independent pathway is activated by TLR3 and TLR4, thus TLR4 is unique in that it can activate signalling via both pathways (Biswas et al., 2007). The best characterised TLR signalling pathway is for TLR4, whose prototypical ligand is LPS (Beutler, 2002). LPS-binding protein (LBP), a soluble plasma protein, binds LPS and the complex then binds CD14, either soluble CD14 found in plasma or the cell surface form found on cell types including monocytes and ovarian GCs (Herath et al., 2007). LPS is then transferred to lymphocyte antigen 96 (LY96), a surface protein which interacts with TLR4. Lipid A (the portion of *E. coli* LPS which binds to TLR4) consists of two phosphorylated glucosamines, acetylated by six lipid chains, five of which bury inside the LY96 hydrophobic binding pocket and the remaining chain interacts with TLR4 (Kawai and Akira, 2010). The two phosphate groups of lipid A form a hydrogen bond with negatively charged residues in TLR4 and LY96, forming a TLR4 dimer connected by LY96, as shown in Fig. 1.8 (Park et al., 2009). If this dimerisation is blocked, signalling via TLR4 cannot occur (Youn et al., 2008).

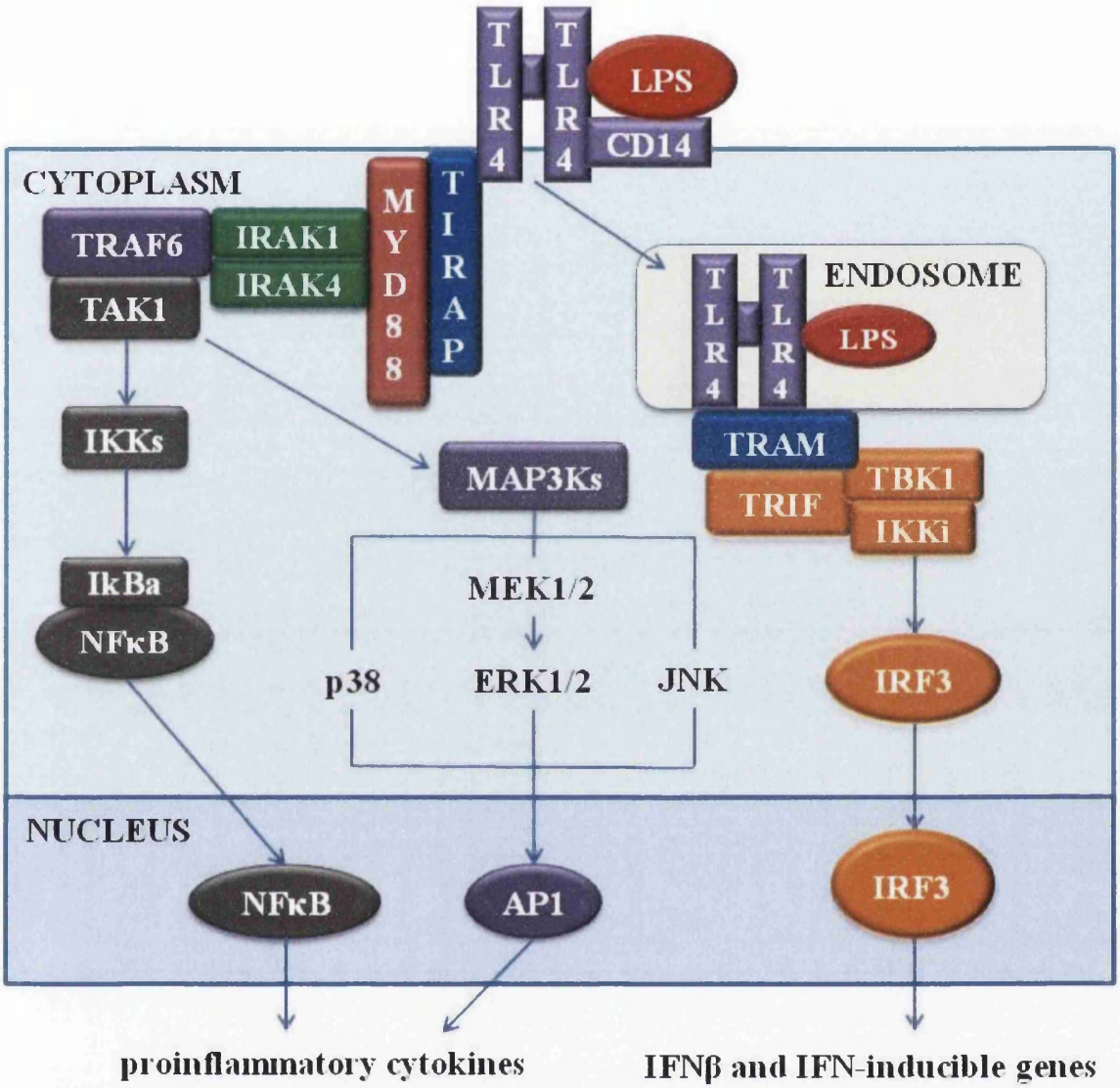


Figure 1.8 TLR4 signalling pathways. LPS binds LBP, which then binds CD14, before being transferred to LY96. This causes TLR4 dimerisation and activation of two signalling cascades. The MyD88-dependent pathway is the first and major pathway, leading to production of pro-inflammatory cytokines such as IL-8, CCL5, IL-6, IL-1β and TNFα. The TLR4-LY96-LPS complex is then endocytosed, recruiting TRAM and TRIF, activating the MyD88-independent pathway, which leads to production of type one interferons and IFN-inducible genes.

1.12.2 MyD88-dependent signalling

The various cellular responses triggered by different TLRs (TLR activation tends to cause production of either type I interferons or inflammatory cytokines) is explained by the presence of Toll-Interleukin Receptor (TIR) domains, found on TLRs and on a number of adapter molecules. One such adapter molecular is MyD88, which activates mitogen-activated protein kinases (MAPKs) and nuclear factor kappa light chain enhancer of B-lymphocytes (NFκB), inducing inflammatory cytokines. This pathway depends on the homophilic interaction of the cytoplasmic TIR domain of the TLR with the C-terminal TIR domain on MyD88. MyD88 is targeted to the plasma membrane where it interacts with TLR4, via TIRAP, which contains a PIP_2 binding domain. If MyD88 is fused to a PIP_2 binding domain TIRAP becomes dispensable, thus TIRAP is only necessary for trafficking of MyD88 to the membrane (Kagan and Medzhitov, 2006).

Upon stimulation, MyD88 recruits IRAK (interleukin-1 receptor associated kinase) through interaction of death domains on both molecules (Neumann et al., 2008). There are four members of the IRAK family, each of which consists of an N-terminal death domain and a central kinase domain (Nguyen et al., 2009). However, only IRAK1 and IRAK4 are catalytically active (Akira et al., 2006). $IRAK1^{-/-}$ knockout mice demonstrate a defective LPS response (Gottipati et al., 2008), whereas $IRAK4^{-/-}$ knockout mice do not respond to PAMPs which stimulate TLR2, TLR3, TLR4, and TLR9 (Suzuki et al., 2002), thus both IRAKs are necessary for a complete response to LPS. IRAK1 is phosphorylated by IRAK4 (Li et al., 2002), leading to association with TRAF6 (tumour necrosis factor receptor associated factor). The TRAF6 molecule is a member of a group of proteins, each consisting of two C-terminal TRAF (tumour necrosis factor associated factor) domains, an N-terminal RING finger, involved in protein-protein interactions, and zinc finger domains (Bradley and Pober, 2001). Upon TLR stimulation, TRAF6 is recruited to the LPS/TLR4/LY96 receptor complex and activated by IRAK1. This complex then interacts with TAK1 (TGF-β (transforming growth factor beta) activated kinase one), before moving away from the membrane bound receptor to the cytoplasm. Then, TAK1 activates MAPKs, which activate a number of transcription factors including AP-1. The RING finger domain of TRAF6 acts as a protein ligase

together with 2 ubiquitin conjugating enzymes to activate IKK (Deng et al., 2000). This causes degradation of the inhibitory protein IκB, allowing translocation of NFκB to the nucleus. The MyD88$^{-/-}$ knockout mice do not produce cytokines in response to LPS (Kawai et al., 1999) and show no cellular response to peptidoglycan (Takeuchi et al., 2000), bacterial DNA (Schnare et al., 2000), flagellin (Hayashi et al., 2001) or imidazoquioline (Hemmi et al., 2002). This indicates that MyD88 is necessary for signalling through TLR2, TLR4, TLR5, TLR7, TLR9 and TLR10 (Hasan et al., 2005). In fact, MyD88 is necessary for signalling through all TLRs except TLR3 (Parker et al., 2007).

1.12.3 MyD88-independent signalling

Although MyD88$^{-/-}$ knockout mice do not produce inflammatory mediators in response to TLR activation with LPS (Kawai et al., 1999), LPS induces delayed activation of NFκB and JNK in MyD88$^{-/-}$ mice, inducing interferon-inducible genes including IP-10, involved in T-cell recruitment (Kawai et al., 2001). This non-classical response to LPS suggested that there may be a MyD88-independent pathway via TLR4, resulting in NFκB activation. In addition to the pathway explained above, LPS also signals through the MyD88-independent, or TRIF/TRAM (TIR domain-containing adaptor inducing IFN/Trif-related adapter molecule) pathway. The TRIF/TRAM pathway activates NFκB and MAPK pathways through activation of TAK1 by a multiprotein complex recruited by TRIF, in a similar way to the MyD88-dependent pathway.

Contrasting to the MyD88-dependent pathway, LPS activation of the TRIF/TRAM pathway also leads to activation of IRF-3, a transcription factor which induces interferon beta (IFN-β) (Toshchakov et al., 2002). TRIF recruits IκB kinases (IKK) including TBK1 and IKKi, which induce IRF-3 phosphorylation, inducing nuclear translocation of IRF3, where it induces type I interferons. TRIF has been shown to be necessary for MyD88-independent signalling via TLR3, as siRNA knockdown of TRIF causes impairment of IFN-β expression (Takeda, 2005). Although in TRIF$^{-/-}$ knockout mice treated with LPS there is little knockdown effect in NFκB activation, it appears that the true effect of TRIF$^{-/-}$ knockout is masked by the early production of NFκB by the MyD88-dependent pathway (Yamamoto et al., 2003).

1.12.4 Cytokines and chemokines

Signalling through TLRs activates both NFκB and AP-1, regardless of the stimulated receptor, resulting in an inflammatory response characterised by increased production of cytokines, including IL-1β, IL-6 and tumour necrosis factor alpha (TNFα), and chemokines (CCL5 and IL-8). Signalling through the TRIF/TRAM pathway also activates IRF3 and IRF7, resulting in the induction of genes that stimulate T cell responses and an antiviral immune response through interferon production.

Cytokines are small peptides, which are involved in a number of processes, including differentiation, cell survival and immunomodulation. Interleukins represent a large family of cytokines, of which IL-6 is classically produced through TLR activation. Interleukin 6 has a wide repertoire of actions, including aiding the adaptive immune response through induction of B-cell differentiation (Urashima et al., 1996) and stimulation of antibody secretion (Dienz et al., 2009). The cytokine is also involved in induction of the acute phase response (Wegenka et al., 1993) and promotes leukocyte recruitment to the area of infection (Romano et al., 1997). Interleukin 1β is a potent cytokine which is important for a number of inflammatory and immunologic processes (Dinarello, 2011). Tumour necrosis factor alpha is another cytokine which is important for the inflammatory response and stimulates phagocytosis by macrophages (Collins and Bancroft, 1992).

There are 4 groups of chemokines, categorised according to the spacing of their first 2 cysteine residues: CC chemokines have adjacent cysteine residues; CXC chemokines have one amino acid between the 2 first cysteines; C chemokines only contain 2 cysteine residues in total, and CX3C chemokines have 3 amino acids between the first 2 cysteines. Chemokines attract immune cells to the area of infection, predominantly neutrophils by IL-8 (Hammond et al., 1995) and T-cell and monocyte/macrophage recruitment by CC chemokines such as CCL5 (Murooka et al., 2008, Chernova et al., 2009, Chan et al., 2012). In addition to functioning as chemoattractants, chemokines also promote immune cell function, for example CC chemokines enhance cytotoxicity of natural killer cells (Robertson, 2002).

1.12.5 Inflammasome activation

In contrast to other cytokines produced through TLR activation, IL-1β is synthesised as an inactive pro-peptide in response to TLR activation. In order for bioactive IL-1β to be secreted, a second signal must be endocytosed by the cell to allow cleavage of the immature protein. This processing is dependent on the NLRP3 inflammasome, a multiprotein complex consisting of NLRP3 (Nod-like receptor pyrin domain containing 3), ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and caspase 1. Although the mechanisms leading to inflammasome activation have not been completely elucidated, it is thought that following internalisation of the second signal, reactive oxygen species are released which liberate an NLRP3 ligand from its inhibitor (Tschopp and Schroder, 2010). This enables NLRP3 to oligomerise and interact with ASC, which recruits pro-caspase 1 to the inflammasome. Clustering of pro-caspase 1 on oligomerised NLRP3 activates caspase 1, which cleaves pro-IL-1β to its secretable form (Tschopp and Schroder, 2010).

1.12.6 Ovulation as an inflammatory process

The acute phase of inflammation is characterised by increased blood flow, increased vascular permeability, leukocyte invasion and release of cytokines, resulting in tissue repair. These traits are also observed in the process of ovulation, during which the ovarian follicle becomes highly vascularised, producing prostaglandins and a hyaluronan-rich matrix, similar to that produced during wound healing (Richards et al., 2008) (Table 1.3). Leukocytes gather in the theca layer of pre-ovulatory follicles (Brännström et al., 1993), which release nitric oxide, causing vasodilation and secreting IL-1β, a cytokine which increases production of collagenases, metalloproteinases and prostaglandins, regulating repair of the ovarian surface (Bonello et al., 1996, Gerard et al., 2004). Interleukin-1 alpha also stimulates the ovarian surface epithelium to increase expression of *IL6* and *IL8*, which increase cellular proliferation, aiding healing of the ovarian surface (Rae et al., 2004, Jabbour et al., 2009). The LH surge induces expression of prostaglandin synthase (Joyce et al., 2001). Prostaglandins are involved in ovarian follicle rupture (Markosyan and Duffy, 2009) and inhibitors of prostaglandin synthesis can block ovulation (Kaur et al., 1986).

Table 1.3 Similarities between inflammation and ovulation (Espey, 1980)

Inflammation	Ovulation
Histamine, bradykinin and prostaglandins cause hyperaemia, vasodilation and oedema	LH surge causes ovarian hyperaemia, vasodilation and oedema
Basophils and leukocytes migrate to area of inflammation	Basophils and leukocytes accumulate in the theca layer prior to ovulation
Proliferating fibroblasts infiltrate areas of inflammation, especially during repair	Theca cell layer around follicle contains fibroblasts, which proliferate prior to follicle rupture
cAMP mediates inflammation and leads to production of prostaglandins	LH surge stimulates cAMP in follicle cells, leading to steroidogenesis and prostaglandin production
Histamine increases vascular function	Histamine has significant function
Prostaglandins are important mediators of inflammation	LH surge causes synthesis of PGE, which suppresses ovulation and PGF, which stimulates ovulation
PGE causes vasodilation, increased vascular permeability, enhances hyperaemia and induces collagenase	PGE causes follicular hyperaemia, leads to production of cAMP and stimulates production of plasminogen activator
The serine protease collagenase is activated	The serine protease plasminogen activator is produced in the follicle during ovulation

1.12.7 Ovulation as an innate immune response

It seems clear from the investigations described above that ovulation involves a local inflammatory reaction. However, recent studies have focussed on the similarities between innate immunity and ovulation and the potential role of innate immunity in ovarian physiology, as well as in pathology. The first link between innate immunity and inflammation came from the discovery that Toll, which is involved in embryo development in Drosophila (Anderson et al., 1985, Halfon et al., 1995), is also involved in defence against fungal infection (Lemaitre et al., 1996). A later study found that 18-wheeler, a Toll-like receptor involved in Drosophila's antibacterial response (Williams et al., 1997), is also involved in follicle cell migration and oocyte morphology (Kleve et al., 2006).

In 2006, a murine gene array study discovered a number of genes associated with immune function, which were induced in the COCs of mice that had been stimulated with gonadotrophins to stimulate growth of pre-ovulatory follicles, cumulus expansion and oocyte maturation (Hernandez-Gonzalez et al., 2006). A further paper from the same year identified the expression of Toll-like receptors (and associated proteins) in murine cumulus oocyte complexes at ovulation, finding that the expression of *Tlr4, Tlr8, Tlr9, Cd14* and *MyD88* increased at ovulation *in vivo* (Shimada et al., 2006). This paper also investigated the expression of these proteins during *in vitro* maturation and found that *Tlr4, Cd14* and *MyD88* expression was increased in response to FSH or the EGF-like factor amphiregulin.

Ovulation of a COC depends on formation of the hyaluronan-rich matrix, and hyaluronan has been reported to signal through TLR2 and TLR4. A study using murine COCs found that treatment of the COC with hyaluronic acid fragments, potentially produced through the action of sperm-produced hyaluronidase, increased expression of mRNA encoding cytokines and chemokines, including CCL5 and IL-6 through TLR2 and TLR4, ERK1/2 and p38 MAPK (Shimada et al., 2008), supporting the hypothesis of a role for TLRs in fertilisation. Interestingly, *Il6* mRNA expression is increased during ovulation or during IVM, and IL-6 itself can cause cumulus expansion without the need for FSH or EGF-like factors (Liu et al., 2009). Additionally, this study reported that IL-6 is able to stimulate GVBD of COCs, suggesting that IL-6 may play a role in oocyte maturation.

1.13 Culture Techniques

The above mentioned studies provide some interesting observations. However, although experiments using a murine model allow a greater range of experiments to be carried out, the results do not necessarily correlate with responses in large mammals such as cows or humans (Bilodeau-Goeseels, 2011). As such, it is important to investigate the impact of inflammatory mediators on ovarian cells of clinically relevant species, and the interactions between the endocrine and immune systems in these target animals.

Culture of primary bovine and human cells provides some challenges. The first 2 result chapters of this thesis report on work using primary bovine GCs. Ideally, GCs are cultured in the absence of serum to prevent luteinisation, maintain FSH responsiveness and oestradiol producing capacity (Rouillier et al., 1996). Previously, optimum conditions for serum-free culture of GCs were identified (Gutierrez et al., 1996). However, GCs do not respond to PAMPs when cultured in the absence of serum (at time of experiments, unpublished results from the group; subsequently published in (Bromfield and Sheldon, 2011), potentially due to the need for soluble factors, such as CD14, found in serum. Therefore, GCs were cultured in the presence of serum for up to 96 hours (although in most experiments only for 72) in order to detect cellular responses to TLRs, whilst attempting to minimise luteinisation.

The third results chapter reports on findings using primary bovine COCs and parthenotes. Although producing embryos would have been more biologically relevant, this would have introduced a new variable of sperm to the experiments, which are not free from LPS or other bacterial components (Scott and Smith, 1997, Okazaki et al., 2010).

In the final results chapter, investigations using a human granulosa cell line are described. The COV434 cell line was chosen because it displays many hallmarks of healthy GCs, such as responsiveness to FSH and the ability to produce oestradiol (Zhang et al., 2000). Although using human primary cells would have been interesting, this was not possible due to the poor availability of human ovaries. A positive aspect of using a cell line was that it avoided immune cell contamination associated with human ovarian follicle aspiration (Fedorcsák et al., 2007).

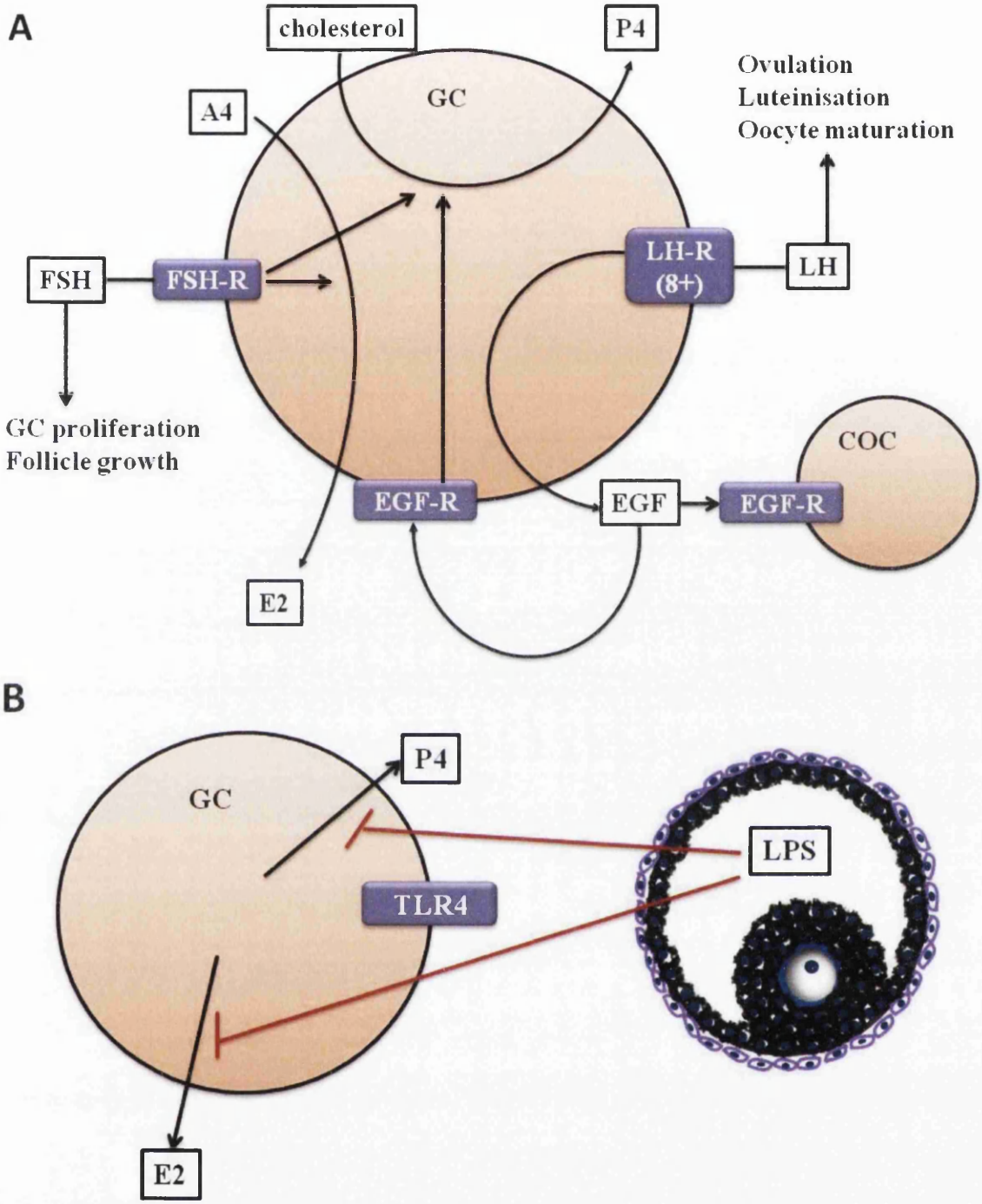


Figure 1.9 A summary of our knowledge of endocrine (A) and innate immune (B) pathways in bovine GCs prior to the thesis. GC: granulosa cell, COC: cumulus-oocyte complex, A4: androstenedione, E2: oestradiol, P4: progesterone, EGF: epidermal growth factor, LH: luteinising hormone, FSH: follicle stimulating hormone, -R: receptor, TLR4: Toll-like receptor 4, LPS: lipopolysaccharide

1.14 Conclusions

Bacterial infection in the reproductive tract can cause uterine disease, a condition which causes subfertility and pain. Uterine disease is most common in humans and cows, with infection after parturition in cattle estimated to cost €1.4 billion in the EU alone per year (Sheldon et al., 2009) and infertility accounting for a third of all bovine culling (Frerking, 1999). The cow provides the only animal system where diseases caused by bacterial infections have consistently been linked to ovarian dysfunction *in vivo*. Understanding the impact of bacterial infection on ovarian health with the view to designing effective treatment or prevention therefore provides an important economical incentive for agricultural and pharmaceutical industries. The first bacterium to invade the uterus post partum is *E. coli,* whose LPS collects in follicular fluid. The immune system senses this LPS, and other bacterial ligands, such as flagellin, through Toll-like receptors, activating intracellular pathways which produce pro-inflammatory cytokines. Prior to the start of this project, little was known about the response of GCs or oocytes to PAMPs or DAMPs, although the biological function of these cells was better understood (Fig. 1.9). The hypothesis tested was that bacterial infections of the bovine reproductive tract perturb ovarian granulosa cell function and the health of the subsequent embryo. The aims of this thesis were:

- To identify whether cells within ovarian follicles express functional Toll-like receptors that detect PAMPs associated with bacteria
- To investigate the pathways involved in granulosa responses to bacterial PAMPs and identify potential therapeutic targets
- To explore the interactions between the innate immune system and endocrine system within ovarian follicles.
- To investigate the impact that follicular fluid LPS contamination may have on oocyte maturation and the subsequent embryo
- To develop a human granulosa cell model suitable for investigating the impact of infections on human ovarian health

Chapter 2

Materials and Methods

2.1 General Materials and Methods

The experiments carried out in the thesis used: primary GCs from emerged (chapter 3) or dominant (chapter 4) bovine ovarian follicles; bovine cumulus-oocyte complexes (COCs) and oocytes (chapter 5), and a human granulosa cell line (chapter 6). The cells were treated with pathogen-associated molecular patterns (PAMPs) associated with bacterial infection and cellular responses evaluated. Cell-free supernatants were collected to measure the accumulation of pro-inflammatory cytokines and hormones, and protein and RNA were extracted from the cells to measure activation of intracellular signals and expression of genes associated with innate immunity or physiology. For work using GCs, it was then investigated whether it was possible to manipulate this response, using biochemical inhibitors or hormones. For experiments using oocytes, egg health was evaluated by examining expression of genes associated with oocyte competence and maturation, and through microscopy.

2.2 Animals

Ovaries were collected from mixed breeds of heifers (Hereford, Aberdeen Angus, Limousin, Charolais, Welsh Black and Belgian Blue breeds; aged 2.2 ± 0.1 years) or dairy cows (Holstein Fresian breeds, aged 3.1 ± 0.4 years) within 15 minutes of killing. The ovaries were transported on ice to the laboratory within 90 min in 75 ml sterile Dulbecco's phosphate-buffered saline (PBS; for formulation see appendix 1) with 1% penicillin/streptomycin (both Sigma, Gillingham, UK) in 150 ml sterile containers.

2.3 Bovine ovary processing

Ovaries were rinsed in 70% ethanol and sterile PBS before processing for mural GCs and cumulus-oocyte complexes (COCs). A sterile 20-gauge needle and endotoxin-free syringe (both BD Medical, Oxford, UK) were used to aspirate emerged (4 to 7.5 mm diameter) or dominant (> 8.5 mm diameter) follicles (Xu et al., 1995, Driancourt, 2001) into 30 ml collection medium (0.5% w/v bovine serum albumin (BSA), 25 mM HEPES, 0.005% w/v heparin, 1% penicillin/streptomycin (all Sigma) in Medium 199) in 90 mm petri dishes (Fisher) warmed to 37°C on a slide warmer. The cells were pooled from between 8 and 20 ovaries for each

experiment. All COCs were collected from the petri dish, using a pulled glass Pasteur pipette (Kimble Chase, Vineland, USA), and maintained in collection medium without heparin prior to culture. The remaining GCs were centrifuged at 700 x g for 10 min (Eppendorf 5810R, Cambridge, UK), re-suspended in 30 ml culture medium (M199 with 10% FCS, 1% penicillin/streptomycin, 1% L-glutamine, 1% ITS; all Sigma) and centrifuged once more at 700 x g for 10 min (Eppendorf 5810R). Granulosa cells were counted using a disposable haemocytometer (Immune Systems, Devon, UK), adjusted to 1.5 x 10^6 cells/ml in culture medium (as above), which was prewarmed to 37°C. The cells were plated in volumes of 2.5 ml, 1 ml or 0.5 ml in 6- , 12- or 24-well plates (TPP, Trasadingen, Switzerland), respectively, for analysis by PCR, Western blot or ELISA, respectively (Fig. 2.1). All cells were cultured at 37°C under 5% CO_2 in an incubator used only for primary cells (chapters 3-5) or cell lines (chapter 6; Binder, Tuttlingen, Germany). Foetal calf serum (FCS; Biosera, Ringmer, UK) was batch-tested using LPS and GCs to ensure minimal stimulation of pro-inflammatory molecules and the same batch of FCS was used throughout the thesis. All medium was filtered through a 0.2 μM filter and stored in the base of the 250 ml filter unit (TPP).

2.4 Preparation of peripheral blood mononuclear cells (PBMCs)

Blood was collected into heparinised tubes from the jugular vein of beef cows within 15 minutes of killing. After transporting to the laboratory at room temperature, the cells and plasma were separated by centrifugation for 10 min at 500 x g (Eppendorf 5810R) with the brake off. The buffy coat was carefully aspirated using a sterile Pasteur pipette and transferred into a sterile 15 ml centrifuge tube. Ten ml of sterile PBS was added to the tube, before centrifugation at 700 x g for 10 min with the brake on. The supernatant was discarded and the cell pellet re-suspended in the residual PBS. Contaminant red blood cells were lysed using 1 ml sterile water, mixed by pipetting for 5 sec, before addition of 10 ml sterile PBS. The cells were pelleted by centrifugation (as above) and washed in PBS again. The PBMCs were then resuspended in 1 ml PBS, counted and adjusted to 1x10^6 cells/ml in PBS.

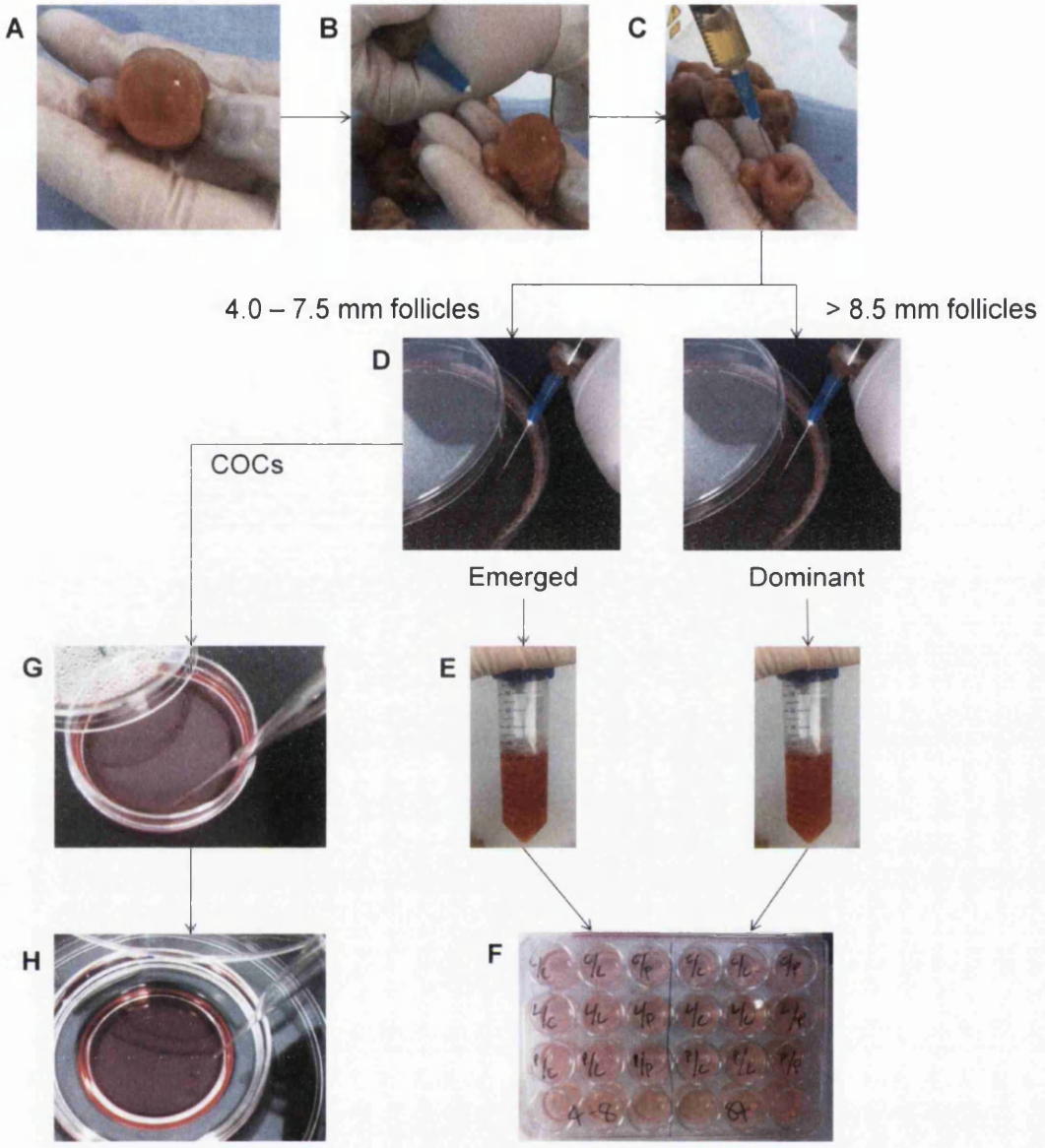


Figure 2.1 Ovaries were processed to extract GCs and cumulus-oocyte complexes for culture. Follicles were graded according to size (A; 4-7.5 mm or >8.5mm) and aspirated using a sterile 20-gauge needle (B). Follicular fluid was examined and only collected if clear (C). The syringe contents were expelled into collection medium into separate petri dishes for cells from emerged and dominant follicles (D). The COCs and denuded oocytes were removed from each pool of cells – for dominant follicle, these were discarded; for emerged follicles, COCs were washed twice in collection medium without heparin (G) and were cultured in organ culture dishes (H). Granulosa cells were transferred to sterile 50 ml Falcon tubes (E) and washed twice with culture medium, before plating in 12-, 24- or 96-well tissue culture dishes (F).

2.5 Granulosa cells from beef heifers represent a suitable model for dairy cows

Post-pubertal non-pregnant heifers from a slaughterhouse were selected for experiments because they do not have gross evidence of disease and are expected to be LPS-naive (Santos et al., 2009). As dairy heifers are rarely culled, it was important to confirm that GCs from readily available beef heifers responded to PAMPs in a similar manner to the dairy cows that are the target for study of uterine disease. Thus, GCs were aspirated from dairy (n = ~35) and beef (n = ~40) heifers and cultured as described above. After an initial 48 h establishment period, they were treated with control medium or medium containing 10-fold increasing concentrations (from 0.001 to 10 μg/ml of ultrapure LPS from *Escherichia coli* 0111:B4 (Invivogen); LPS was used because it collects in follicular fluid in cows with metritis (Herath et al., 2007) and it stimulates increases in production of IL-6 by murine GCs (Gorospe and Spangelo, 1993). After 24 h culture, supernatants were collected and the accumulation of inflammatory cytokines IL-6, IL-1β and the chemokine IL-8 measured by ELISA, as described below (2.19).

Lipopolysaccharide stimulated a dose-dependent increase in accumulation of inflammatory mediators by GCs from beef or dairy heifers (Fig. 2.2). Treatment of beef GCs with concentrations $\geq$ 0.01 μg/ml LPS significantly increased the accumulation of IL-6, IL-1β and IL-8 (Fig. 2.3 A, C, E; $P < 0.001$) compared with untreated cells. Treatment of GCs from dairy heifers with doses $\geq$ 0.001 μg/ml LPS significantly increased accumulation of IL-6 (Fig. 2.3B; $P < 0.05$) and doses $\geq$ 0.1 μg/ml LPS significantly increased accumulation of IL-1β (Fig. 2.3D; $P < 0.05$) compared to untreated cells. There was a trend for GCs from dairy heifers to exhibit a lower response to LPS than GCs from beef heifers, with a significant difference in accumulation between populations for IL-1β ($P < 0.01$) and IL-8 ($P < 0.001$), but not for IL-6 ($P = 0.14$). However, as GCs from both dairy and beef cattle responded to LPS, and IL-6 is the main marker of inflammation used in this work, GCs collected from beef breeds represented a suitable model for investigating the effects of infection on ovarian health. All further experiments with bovine GCs used post-pubertal non-pregnant beef heifers (n = ~600 across all experiments). Each independent experiment used GCs pooled from 6 - 10 animals.

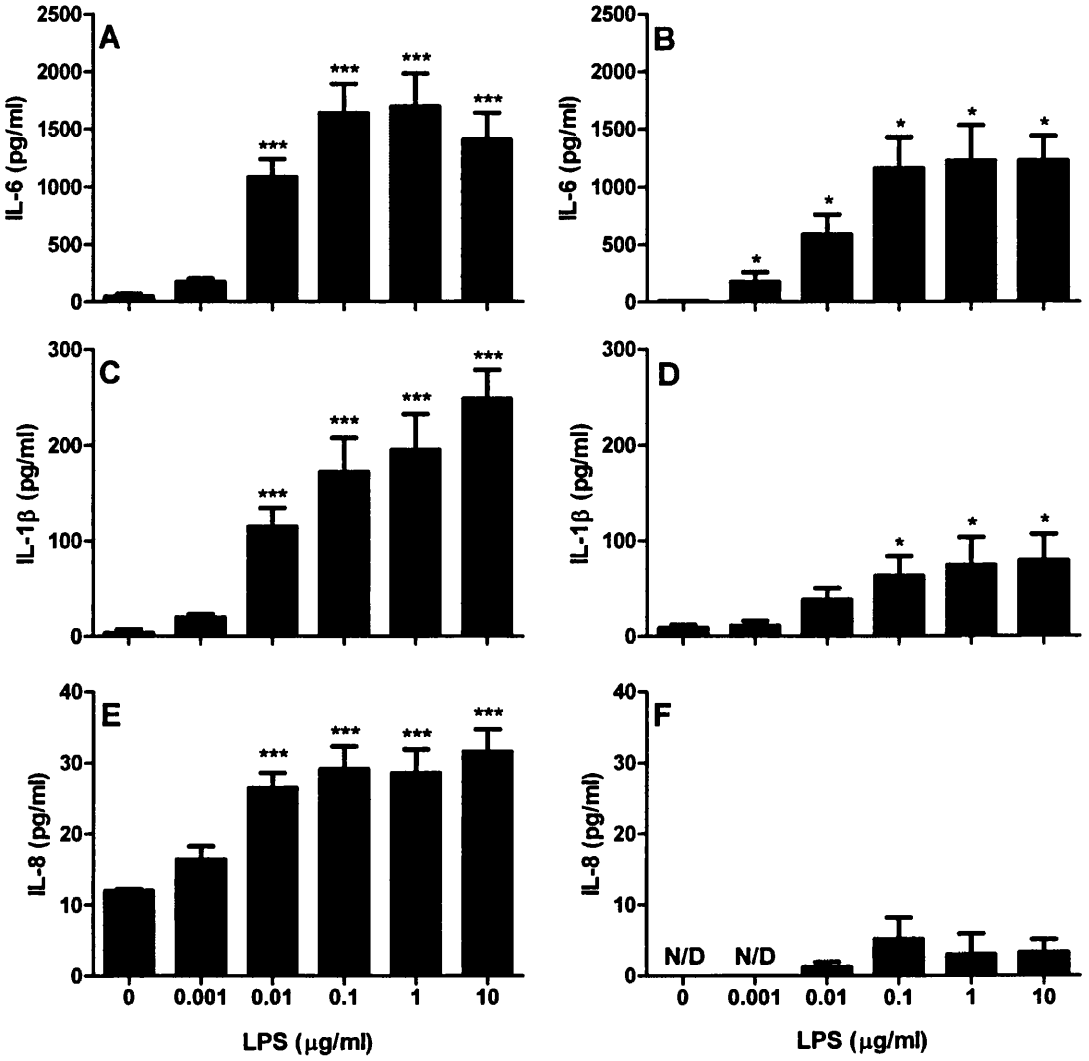


Figure 2.2 Comparison of LPS response by GCs from beef and dairy cows. Granulosa cells were isolated from beef heifer (A, C, E) or dairy cow (B, D, F) emerged ovarian follicles, culture established for 48 h, then treated with control medium or medium containing 0.001, 0.01, 0.1, 1 or 10 μg/ml LPS for 24 h. The accumulation of IL-6 (A, B), IL-1β (C, D) and IL-8 (E, F) was measured in culture supernatants by ELISA. Data are presented as mean + SEM and represent 4 to 5 independent experiments. Data from beef cattle were analysed by ANOVA with Dunnett's pairwise multiple comparison t test, using square-root normalised data (IL-6 and IL-1β) or $\log_{10}$ normalised data (IL-8); data from dairy cows was analysed using Mann-Whitney U test; values differ from control, * $P < 0.05$, *** $P < 0.001$.

2.6 Determining the optimum duration of treatment for granulosa cell culture

To determine the optimum duration of treatment for examining protein responses, GCs were isolated from emerged follicles and treated with control medium or medium containing 1 μg/ml LPS for 0, 2, 4, 8, 24 or 30 h. The accumulation of IL-6, IL-1β and IL-8 measured in the supernatant by ELISA (Fig. 2.3A, C, E). There was a significant increase in IL-6 concentration from 6 h and in IL-1β and IL-8 accumulation from 24 h. Thus, 24 h was chosen as the appropriate treatment duration for analysis by ELISA.

To determine the optimum treatment duration for RNA analysis, GCs were treated with control medium or with medium containing 1 μg/ml LPS for 1, 3 or 5 h (Fig. 2.3B, D, F). An increase in expression of *IL6, IL8* and *IL1B* was evident from 1 h to 5 h, and so 3 h exposure was chosen for future experiments.

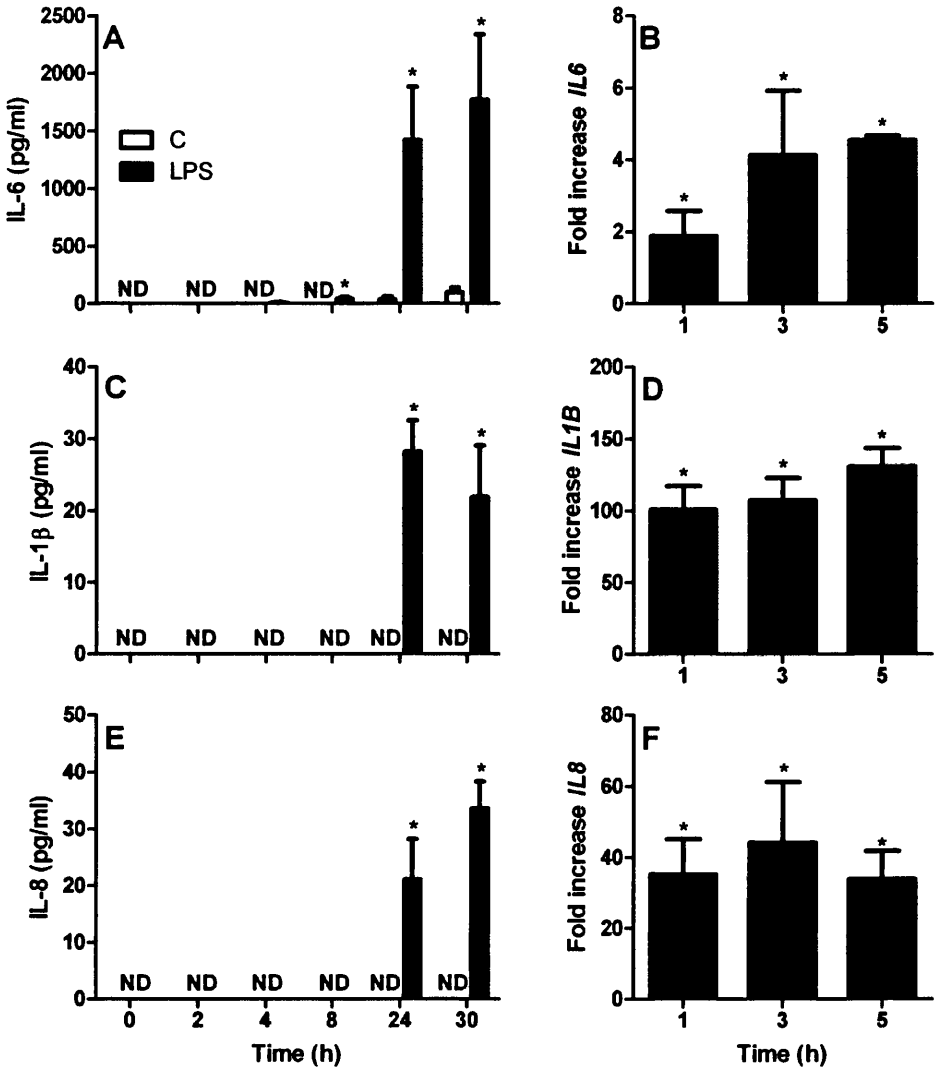


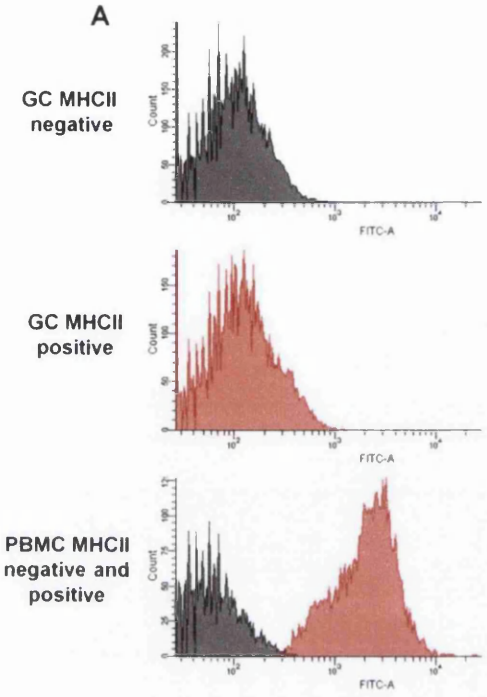
Figure 2.3 Determination of optimum treatment duration. Granulosa cells from emerged follicles were equilibrated for 48 h, then treated for 0, 2, 4, 8, 24 or 30 h with control medium ('C') or medium containing 1 μg/ml LPS. Supernatants were collected and the accumulation of IL-6 (A), IL-1β (C) and IL-8 (E) measured by ELISA. Data presented as mean + SEM and represent 4 independent experiments. The GCs were also treated for 1, 3 or 5 h with control medium or medium containing 1 μg/ml LPS and expression of *IL6* (B), *IL1B* (D) and *IL8* (F) was measured by qPCR (normalised to *ACTB*) and expressed as fold change relative to control. Data are presented as mean + SEM and represents 3 independent experiments. Values differ from 0 h (A, C, E) or control (B, D, F) by Mann Whitney U test, * $P < 0.05$

2.7 Validation of precision of granulosa cell isolation

To ensure that any observations from GCs were not associated with contaminating immune cells, the granulosa cell preparations were examined for absence of immune cell markers using 2 different methods. First, after isolation and prior to any culture, GCs and peripheral blood mononuclear cells (PBMCs) were stained for the lymphocyte and antigen-presenting cell marker MHCII. Flow cytometry analysis of 10,000 cells (for method see 2.13) showed that 0% cells in the population of GCs from emerged and dominant follicles expressed MHCII, compared to 33% of cells in the blood cell population (Fig. 2.4A). The expression of *MHCII* and cluster of differentiation 45 (*CD45*), a marker of leukocytes, neutrophils and monocytes (Loken et al., 1990), was then measured in these cells by qPCR (for method see 2.20). There was no *MHCII* or *CD45* mRNA detected in RNA from GCs isolated from emerged or dominant follicles compared to high expression in the mRNA of PBMCs collected from the buffy coat (Fig. 2.4B). Expression of anti-Müllerian hormone (*AMH*), a marker of GCs was also measured, with high expression in GCs from emerged follicles, lower expression in GCs from dominant follicles and no detectable expression in cells from the buffy coat (Fig. 2.4B).

As follicles emerge during follicle development, they express the FSH receptor (*FSHR*), but only express the LH receptor (*LHCGR*) on reaching dominance. In order to confirm the precision of GC isolation from emerged vs dominant follicles, the GC expression of *FSHR*, *LHCGR* and *CYP19A1* was measured by qPCR. The GCs from emerged and dominant follicles both expressed *FSHR* (Fig. 2.4B); GCs from dominant follicles had a greater expression of *CYP19A1* (Fig. 2.4B), and only GCs from follicles over 8.5 mm in diameter expressed *LHCGR* (Fig. 2.4B), confirming the follicle diameters chosen discriminated between emerged and dominant follicles.

A



B

Gene	Emerged	Dominant	MNC
CD45	0.0	0.0	1.0
MHCII	0.0	0.0	1.9
AMH	5.6	2.2	0.0
FSHR	6.4	6.9	0.0
LHCGR	0.0	3.6	0.0
CYP19A1	0.1	1.9	0.0

Figure 2.4 Granulosa cell preparations were free from immune cells. Granulosa cells were isolated and examined immediately for presence of contaminating MHCII expressing immune cells. Peripheral blood mononuclear cells were used as a positive control. (A) Flow cytometry histograms showing unstained population of GCs or blood-derived mononuclear cells in grey and cells positive for MHCII in red. Cell purity of each cell type was evaluated by FACS on 5 separate occasions; a representative histogram is shown. (B) Mean gene expression relative to *ACTB* (from 3-4 experiments) of immune or granulosa cell markers in GCs from emerged or dominant follicles or PBMCs.

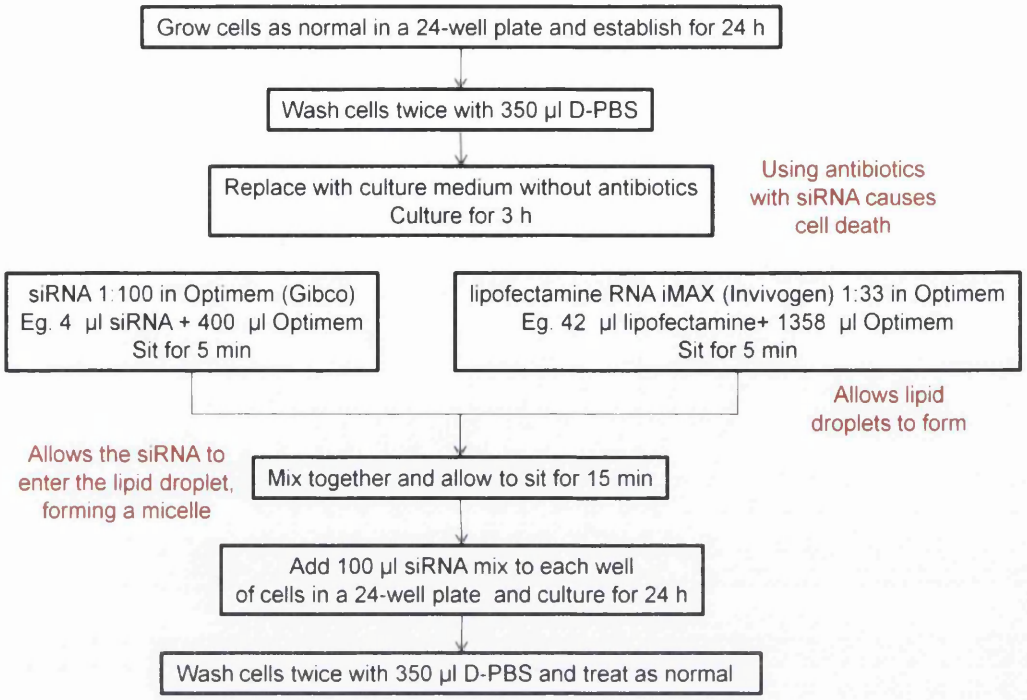
2.8 Granulosa cell culture and treatments

Granulosa cells were cultured and treated with PAMPs (LPS, Pam3CSK4 or flagellin) or a DAMP (hyaluronic acid), as described for each experiment. Ultrapure LPS from *Escherichia coli* 0111:B4 (Invivogen) was used from 0.001 to 10 μg/ml; the synthetic lipopeptide Pam3CSK4 (PAM; Invivogen) was used from 0.001 to 10 μg/ml, and ultrapure flagellin from *Salmonella typhimurium* (FLA; Invivogen) was used from 0.0001 to 1 μg/ml. Ultra-low molecular weight (ULMW HA) and high molecular weight (HMW HA) hyaluronan produced from *Streptococcus pyogenes* (R&D Systems) were used from 0.1 to 1000 μg/ml. Prior to treatment, cells were washed in 0.3 ml sterile PBS. Treatments were diluted to the required concentration immediately before use in warm culture medium and vortexed before use. Some experiments used short-interfering RNA (siRNA; designed and validated for off-target effects using Dharmacon siDESIGN centre, Thermo Scientific), as shown in Fig 2.5 or small molecule inhibitors (Table 2.1). After treatment, supernatants were stored at -20°C in a microcentrifuge tube rack (Alpha Labs, Hampshire, UK) for analysis of protein by ELISA. Cells were washed twice in 0.3 ml sterile PBS, before harvesting for RNA or protein analysis by PCR or Western blot respectively, or estimating cell viability using an MTT assay.

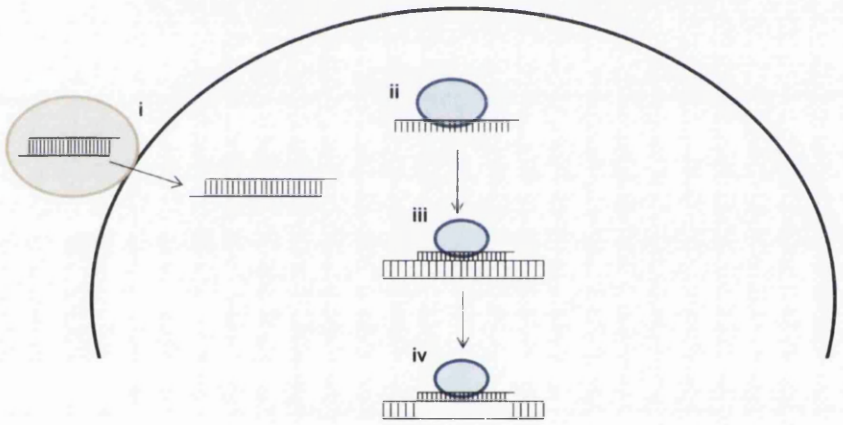
Table 2.1 Small molecule inhibitors used to examine active pathways.

Target	Name and code (all Calbiochem)	Working concentration (μM)
NFκB	NFκB activation inhibitor Insolution	0.0004 - 40
PKC	RO-31-7549	0.001 - 100
p38 MAPK	MAPK inhibitor SB230580	0.001 - 100
MEK	MEK inhibitor U0126	0.0001 - 10
JNK	JNK inhibitor II	0.005 - 500
ERK (chapter 6)	ERK activation inhibitor peptide 1	10

A



B



C

Target	Sense	Antisense
TLR2	GGACAGAAUUAGACACCUAUU	UAGGUGUCUAAUUCUGUCCUU
TLR4	GAGUAUAUCUUUAGGAAGUUU	ACUUCCUAAAGAUAUACUCUU
Scramble	ON-TARGETplus Non-targeting siRNA #1 (Dharmacon)	

Figure 2.5 Granulosa cells were treated with siRNA. (A) Flow plan showing schedule of experiment. (B) (i) Micelles fuse with the cell's plasma membrane and the siRNA enters the cell by endocytosis. (ii) Argonaute binds the guide strand of the siRNA and the other strand is degraded. (iii) The siRNA, argonaute and other proteins forms RISC (RNA-induced silencing complex) and binds to the complimentary region on the mRNA. (iv) Argonaute catalyses the cleavage of the mRNA which is then degraded. (C) Sequences for siRNA used in this project (Dharmacon).

2.9 Cumulus-oocyte complex culture, treatment and staging

The cumulus-oocyte complexes (COCs) were collected from the petri dish using a pulled glass Pasteur pipette and washed twice in collection medium without heparin, before transferring 15-20 COCs into 1 ml culture medium (0.25 mM sodium pyruvate and 1 μg/ml oestradiol in GC culture medium) in round bottom organ culture dishes (BD Falcon, Franklin Lakes, USA). For some cultures, medium was supplemented with 2.5 μg/ml bovine FSH and 10 μg/ml bovine LH (courtesy of AF Parlow, National Hormone and Peptide Program, Torrance, USA). The COCs were cultured for 3 or 24 h in control medium or medium containing 1 or 10 μg/ml LPS or PAM and supernatant was collected for analysis by ELISA. The COCs were then washed once in 300 μl PBS and lysed in buffer RLT (Qiagen, Crawley, UK) for analysis by multiplex PCR (see 2.21). The COCs treated for 24 h were examined prior to lysis for cumulus expansion, staging from 0 to 4, as shown in Fig. 2.6, using previously reported criteria (Vanderhyden et al., 1990).

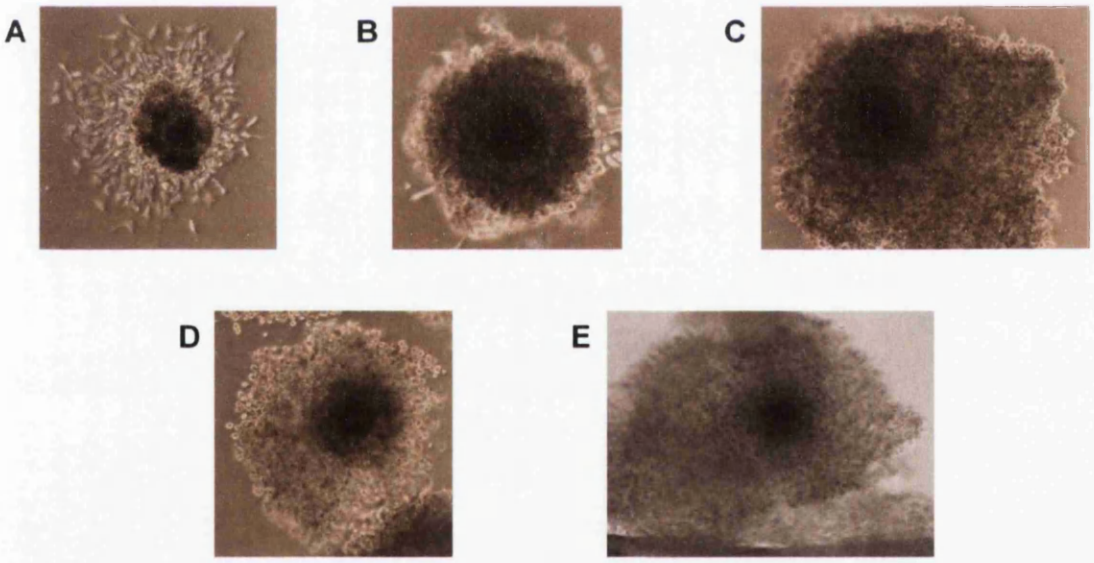


Figure 2.6 Cumulus-oocyte complexes were staged for cumulus expansion. After 24 h in-vitro maturation, COCs were staged using a 5-point scale. Stage 0 COCs showed no response, with cumulus cells adhered to the plastic (A); stage 1 COCs had yet to expand, with the COC still in a firm sphere (B); the outer layer of stage 2 COCs had started to expand and the cells glistened more (C); stage 3 COCs were mostly expanded except the innermost cumulus layer (D); stage 4 COCs were fully expanded and spongy both in sight and when handling (E).

2.10 Parthenote culture

Prior to starting parthenote culture, mineral oil (Sigma) was washed in a sterile 500 ml plastic bottle (500 ml oil plus 50 ml GC culture medium) by stirring for 48 h, allowing the mixture to separate, and then removing the oil. The oil was then washed once again for 48 h, before filtering the separated oil though a 0.2 μM sterile filter unit (Nalgene) and storing the oil in the filter base at room temperature in the dark.

Cumulus-oocyte complexes were aspirated (as described above) and matured in control medium (containing LH and FSH) or medium containing 10 μg/ml LPS or PAM for 24 h at 37°C under 5% CO_2. On the day of *in vitro* maturation (IVM), embryo culture medium (0.25 mM sodium pyruvate, 8 mg/ml BSA, 10% L-glutamine, 10% penicillin/streptomycin in medium 199) and mineral oil were equilibrated at 37°C in defined atmosphere of 5% CO_2, 90% N_2 and 5% O_2 overnight in an incubator used only for parthenote culture (Galaxy 48R; New Brunswick, Enfield, USA). The next day, for each treatment, one culture dish was set up with three 50 μl wash drops and one 25 μl culture drop (all embryo culture medium) completely covered and surrounded with mineral oil. A dish containing DMAP (6-dimethylaminopyridine; Sigma) was also made with two 50 μl wash drops (both embryo culture medium) and two drops of 2 mM DMAP in embryo culture medium (one 50 μl wash drop and one 25 μl culture drop) under mineral oil.

After 24 h IVM, COCs were stripped of their cumulus cells in hyaluronidase (Sigma; 300 μg/ml in 1 ml medium – direct into culture dish) for 4 min at 37°C. In order to remove remaining cumulus cells, oocytes were stripped by manual pipetting through a pulled glass pipette. The oocytes were then washed 3 times in collection medium before treating with 5 μM ionomycin (Calbiochem) in collection medium for 4 min. This ionomycin treatment activated calcium oscillations within the egg, similar to those observed at fertilisation (Ross et al., 2008). This transient increase in calcium leads to the cortical reaction and resumption of meiosis (Raz et al., 1998).

Following ionomycin treatment, the activated oocytes were transferred into the DMAP culture dish, washed twice in culture medium, once in DMAP and then cultured in DMAP for 4 h. The DMAP treatment inhibited protein phosphorylation,

downregulating activity of maturation-promoting factor (Ross et al., 2008) and inducing mitosis (De La Fuente and King, 1998). After DMAP treatment, oocytes were transferred to the culture dish and washed 3 times in culture medium before culturing for 42 h. After 42 h culture, parthenotes were examined and the number of cells recorded and cells lysed with 75 µl buffer RLT for RNA analysis.

2.11 Culture of a human granulosa cell line

The COV434 cells (a kind gift at passage 28 from Dr P I Schrier, Leiden University Medical Centre) were received in our laboratory and used in the present work with the first culture denoted as "passage 1". All experiments used cells from passage 3 to 12 and were performed on at least 3 occasions. The COV434 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK), supplemented with 10% FCS (Biosera), 1% antibiotic/antimycotic (ABAM; Sigma) and 1% L-glutamine (Sigma). Medium was changed every 2 to 3 days, using 4 ml 2.5% trypsin in EDTA (Sigma) to re-suspend the cells when confluent, before splitting 1 flask into 3. When cells were to be treated, 4 ml PBS was used to wash the cells. Then, 2 ml Accutase (Gibco), warmed to 37°C was used to detach the cells from the flask; cells were counted using a haemocytometer and diluted to the required seeding density. An initial experiment tested the response of COV434 cells seeded at $1x10^5$, $2x10^5$, $5x10^5$, $1x10^6$, $2x10^6$ and $4x10^6$ cells/ml to 1 µg/ml LPS. Subsequent experiments used $5x10^5$ cells/ml, as this was the highest density able to be used without medium exhaustion occurring during 48 h culture. The cells were seeded into a 12-well or 24-well plate in 1 ml medium/well, for mRNA or protein analysis, respectively and equilibrated for 24 h at 37°C under 5% CO_2. Medium was then aspirated from each well and 1 ml treatment or control medium added.

For some experiments, cells were cultured in the presence of 1 ng/ml or 100 ng/ml FSH (courtesy of AF Parlow, National Hormone and Peptide Program, Torrance, USA) and 2864.1 ng/ml androstenedione (Sigma), concentrations previously used in the literature (Zhang et al., 2000). Other treatments including PAMPs and small molecule inhibitors are described in detail in chapter 6. After the appropriate treatment duration (as described in chapter 6), supernatants were removed and stored at -20°C, and the cells were either discarded or stored at -80°C in buffer RLT for RNA extraction.

2.12 Culture of THP-1 human monocytic cell line

The THP-1 cell line was derived from peripheral blood of a patient with acute monocytic leukaemia (Tsuchiya et al., 1980). These cells are cultured as monocytic cells in suspension but readily differentiate into a macrophage-like cell after treatment with phorbol-12-myristate-13-acetate (PMA). In chapter 6, THP-1 cells were used as a positive control to test human cellular responses to PAMPs. The THP-1 cells were obtained from American Type Culture Collection (ATCC, Teddington, UK) and were cultured in a THP-1 medium composed of RPMI 1640 medium (Sigma), supplemented with 10% FCS and 1% ABAM (Sigma) at 37°C with 5% CO_2 in air. Cells were split 1 flask into 3 flasks of pre-warmed, pre-gassed medium twice a week. When cells were to be treated, 10 ml confluent cell suspension was diluted with 15 ml normal medium plus 25 µl filter sterilised PMA (Sigma) at 5 ng/ml to differentiate the cells into a macrophage phenotype over 24 h, in a 12- or 24-well plate with 1 ml/well at a cell density of $2x10^5$ cells/ml. They were then washed in 0.5 ml PBS (Sigma) and incubated for 3 h in control THP-1 medium. Medium was then aspirated from each well and 1 ml treatment or control medium added. Treatments are described in detail in chapter 6 but included PAMPs and the DAMP hyaluronic acid. After treatment, supernatants were removed and stored at -20°C and cells were either discarded or stored at -80°C in buffer RLT for mRNA analysis.

2.13 Flow cytometry

Flow cytometry is a technique that enables the analysis of many single cells simultaneously. Cells in suspension are run through the flow cytometer and hydrodynamically focussed through a small nozzle, forcing single cells past the laser. As the light from the laser hits the cell, light is reflected in a number of directions and is then detected. Forward scatter correlates to the size of the cell and side scatter to the complexity of the cell (Kim et al., 2003). Any fluorophores associated with the cells are excited by a particular wavelength of light provided by the lasers, and emitted light of can be detected using filters for discrete wavelengths. Thus through flow cytometry, individual cells can be analysed for size, complexity, and the binding of a particular fluorochrome-conjugated antibody.

Granulosa cells or PBMCs were isolated (as described above) and adjusted to $4x10^5$ cells/ml in PBS. They were then centrifuged at 500 x g for 7 min and the supernatant removed, prior to incubating in the dark for 30 min in 1 ml PBS (unstained) or 1 ml DRAQ5 (a kind gift from Dr Cathy Thornton) at 0.5, 2.0 or 5.0 μM in PBS; DRAQ5 is a membrane-permeant fluorescent dye that binds DNA and can be used in living cells (Martin et al., 2005). Here, it was used prior to experiments using flow cytometry as an output, in order to gate for live DNA-containing cells. Cells were analysed using FACSAria (BD Biosciences), which enables excitation via a blue (488 nm), red (638 nm) or violet (405 nm) laser and emission to be detected via various fluorochrome-dependent filter sets.

Cells were first analysed by cell size and complexity. A plot of forward scatter versus side scatter was created using the unstained cells, and gates were drawn around distinct cell populations (see Fig. 2.8A, B and 2.9A). The cells stained with DRAQ5 were then examined, to verify that the gating was around cells with a nucleus, rather than cellular debris. At 2.0 μM, DRAQ5 had good discrimination from background, so using this histogram, the cells of interest were gated and highlighted on the forward scatter versus side scatter plot, allowing a gate to be drawn around cells of interest. For future experiments, this gate was used to identify the cell populations of interest, with 10,000 events of interest recorded.

To stain for MHCII, untreated cells were washed twice in PBS; then incubated for 30 min with mouse anti-ovine MHCII (AbD Serotec, Oxford, UK) diluted 1:100 in FACS buffer (0.2% BSA in PBS). Cells were washed twice in 2 ml FACS buffer (500 x g for 5 min) and incubated at 4°C for 30 min with Alexa Fluor-488 goat anti-mouse (Sigma) diluted 1:500 in FACS buffer. Cells were washed twice with 2 ml FACS buffer before analysis by FACSAria.

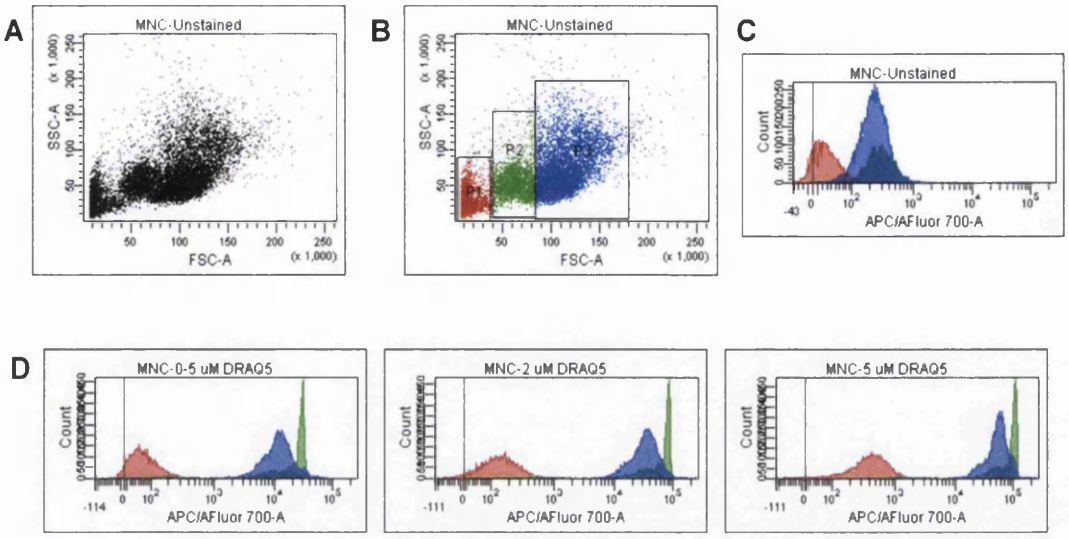


Figure 2.7 Peripheral blood mononuclear cells were stained with the nuclear stain DRAQ5 to identify the cell population of interest. A plot examining side scatter versus forward scatter for unstained PBMCs showed three distinct cell populations (A), which were gated as P1, P2 and P3 (B). The unstained cells were examined for auto-fluorescence with excitation at 638 nm and emission detected using 690 nm long pass filter and 710/40 bandpass filter (C). Cells stained with DRAQ5 (D; 0.5, 2.0 or 5.0 μM from left to right) were examined. The three gated populations showed different characteristics, with P1 (shown in red) showing little nuclear staining, a small size and little complexity, thus likely to be debris; P2 (shown in green) were smaller and less complex than events in the P3 gate (blue), yet had a larger amount of nuclear material, suggesting that cells in P2 were lymphocytes and cells in P3 were the mononuclear cells of interest.

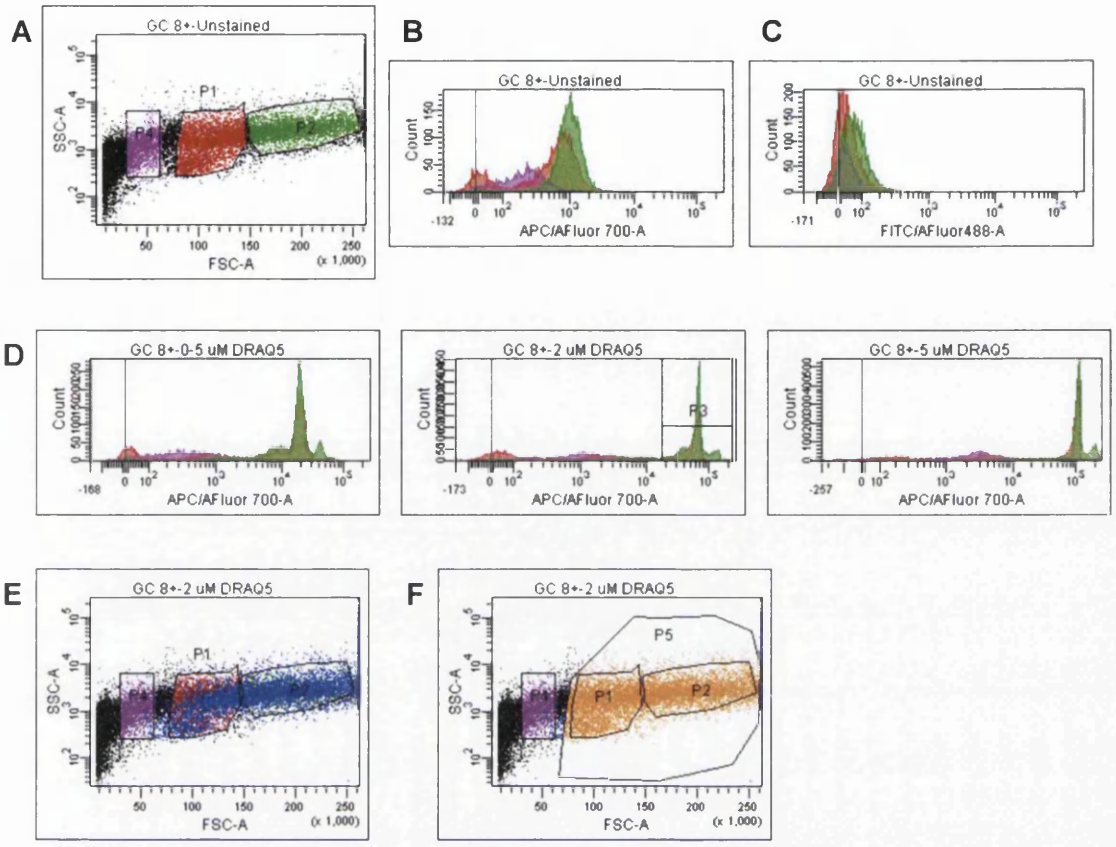


Figure 2.8 Granulosa cell populations were stained with DRAQ5 to identify the cell population of interest. A plot examining side scatter versus forward scatter for unstained GCs showed a number of populations (A). Gates were drawn around predicted populations of debris (P4), single cells (P1) and multiple cells (P2). The unstained cells auto-fluoresced with excitation at 638 nm and emission detected using 690 nm long pass filter and 710/40 bandpass filter (B). However, there was no auto-fluorescence off the blue laser (C), so future experiments preferentially used FITC-conjugated antibodies. Cells stained with DRAQ5 (D; 0.5, 2.0 or 5.0 μM from left to right) were examined and 2.0 μM staining used to gate for the cells of interest. These events were shown on the original forward versus side-scatter plot (E; in blue) and used to gate around cells of interest for future experiments (F; shown in orange).

2.14 RNA Extraction

Total RNA was extracted from GCs, COCs, parthenotes or PBMCs using a Qiagen RNeasy Mini (GCs and PBMCs) or Micro Kit (COCs and parthenotes; both Qiagen, Crawley, UK). Extractions were performed in an RNA-only area of the lab, precleaned with RNase Zap wipes (Ambion, Austin, USA), using DNase and RNase-free filtered pipette tips (Starlab, Milton Keynes, UK). For treated GCs, cells were washed twice at room temperature using 350 µl sterile PBS and then 600 µl buffer RLT (Qiagen) was used to lyse the cells in each well of a 6-well tissue culture plate. The cells were removed from the plate using a sterile cell scraper (Greiner BioOne, Stonehouse, UK). For GCs or PBMCs, when RNA was extracted immediately after cell isolation, cells were pelletted through centrifugation, washed in PBS and 600 µl buffer RLT used to lyse the cells. Cumulus-oocyte complexes were transferred to a 0.5 ml tube, washed in 300 µl sterile room temperature PBS and lysed in 75 µl (for fewer than 15 COCs) or 350 µl (for more than 15 COCs) buffer RLT.

Lysates were transferred to 1.5 ml tubes (Qiagen) and homogenised by passing the suspension through a 21-gauge needle fitted to a sterile syringe (BD Biosciences, Oxford, UK) 20 times, or, for cumulus-oocyte complexes, centrifuging at 8000 x g for 2 min through a QIAshredder column (Qiagen). One volume of 70% molecular grade ethanol (Sigma) was then added to the lysate and mixed by pipetting.

2.14.1 RNeasy Mini kit

Up to 700 µl of the sample was transferred to an RNeasy spin column (Qiagen) in a 2 ml collection tube, centrifuged (Eppendorf 5415 R, Cambridge, UK) for 15 s at 8000 x g and the flow-through discarded. If any sample remained, it was centrifuged through the same spin column, after discarding the first flow-through. The spin column membrane was washed with 700 µl buffer RW1 (Qiagen) and centrifuged for 15 s at 8000 x g and the eluate discarded. Five hundred µl buffer RPE (Qiagen) was used to wash the membrane twice, once with centrifugation for 15 s and the second time for 2 minutes, both times at 8000 x g, with the flow-through discarded after each centrifugation. The spin column was then dried by placing it in an empty 2 ml collection tube (Qiagen) and centrifuging for 1 min at 8000 x g.

The spin column was placed in a new 1.5 ml collection tube and 40 µl RNase-free water (Qiagen) pipetted directly onto the membrane. After a final centrifuge for 1 min at 8000 x g, the tubes containing RNA were placed on ice. The RNA was either kept on ice and used immediately or stored at -80°C and discarded if freeze-thawed more than twice.

2.14.2 RNeasy Micro kit

Up to 700 µl of the sample was transferred to an RNeasy MinElute spin column (Qiagen) in a 2 ml collection tube and centrifuged for 15 s at 8000 x g and the flow-through discarded. The spin column membrane was washed with 700 µl buffer RW1 (Qiagen) and centrifuged for 15 s at 8000 x g and the eluate discarded. The membrane was then washed with 500 µl buffer RPE (Qiagen), centrifuging for 15 s at 8000 x g, followed by washing with 500 µl 80% molecular grade ethanol for 2 min, with the flow-through discarded after each centrifugation. The spin column was then dried by placing it in an empty 2 ml collection tube (Qiagen), opening the spin column lid and centrifuging for 5 min at full speed. The spin column was placed in a new 1.5 ml collection tube and 14 µl RNase-free water (Qiagen) pipetted directly onto the membrane. After a final centrifuge for 1 min at 8000 x g, the tubes containing RNA were placed on ice. RNA was either kept on ice and used immediately or stored at -80°C and discarded if freeze-thawed more than twice.

2.15 RNA quantification

The quantity of RNA was measured using the Nanodrop ND 2.0 spectrophotometer (Nanodrop products, Wilmington, USA). The spectrophotometer was first cleaned using water and a 1 µl sample of water pipetted onto the lower optic surface, before initialising the Nanodrop through the instrument's software. The constant for RNA was selected in the computer program and a 1 µl sample of water was then used to make a blank measurement, by pipetting it onto the lower optic surface, closing the pedestal and clicking on 'blank' on the computer program. The pedestals were then wiped and 1 µl of each sample in turn loaded onto the spectrophotometer and the amount of RNA quantitated by selecting 'measure.' The optical surfaces on the upper and lower pedestal were wiped with clean lens paper after each sample had been read, and the machine cleaned after all samples had been read using water. Samples were discarded if they contained under 40 ng/µl RNA or if the absorbance ratio at 260/280 was not between 1.8 and 2.2, as pure RNA has an absorbance ratio at 260/280 of about 2 with a ratio of greater than 1.8 indicating acceptable RNA quality (Thermoscientific, 2011).

2.16 cDNA synthesis

The RNA and DNA were handled in a dedicated laminar flow hood, using sterile filter pipette tips (Starlab), autoclaved microfuge tubes and pipettes dedicated for this purpose. The hood and items used for PCR were cleaned with 70% ethanol prior to use. The QuantiTect Reverse Transcription kit (Qiagen) was used to synthesise cDNA. This kit contains gDNA wipeout buffer, reverse transcriptase (RT), Quantiscript RT buffer, RT primer mix and RNase-free water. The contents were kept at -20°C and defrosted at room temperature, except the RT which was defrosted on ice. When defrosted, these solutions were mixed by flicking the tubes and centrifuged briefly to collect any residuals on the tube, then kept on ice. Genomic DNA was removed using 2 µl of gDNA wipeout buffer per reaction, plus up to 1 µg of RNA. The volume was then made up to 14 µl with RNase-free water in a thin walled 200 µl tube. This mixture was mixed and incubated for 2 min at 42°C using a TC-312 Thermocycler (Techne, Stone, UK) and placed immediately on ice. Whilst gDNA elimination occurred, the RT master mix was prepared, consisting of 1 µl of RT, 4 µl of RT buffer and 1 µl of the RT primer mix per reaction. Six µl of

this master mix was added to each tube of RNA (after gDNA elimination). The tubes were then mixed, pulsed in a microcentrifuge and heated to 42°C for 15 min and 95°C for 3 min using the thermocycler.

2.17 Polymerase Chain Reaction

Polymerase chain reactions (PCR) were carried out in 0.2 ml thin-walled PCR tubes or in a 96-well 0.2 ml PCR plate (Sarstedt, Nümbrecht, Germany). Primers had either previously been cited in the literature or were designed using NCBI PrimerBlast (conditions: primer length 15 - 25 bp, GC content 20 - 80%, melting temperature 57 - 63°C, PCR product length 70 - 1000 bp) and ordered from Sigma Genosys (see Table 2.2). Primers were centrifuged before opening and reconstituted in RNase-free water to a concentration of 100 µm before aliquoting and storing at -20°C with each aliquot discarded if freeze-thawed more than twice. All primer sequences were validated using NCBI BLAST. For non-quantitative PCR, Biomix Red (Bioline, London, UK) was used, a premixed 2x solution containing Taq polymerase and a red dye allowing direct loading of PCR products onto a gel. Each reaction contained 25 µl Biomix red, 0.5 µl each primer, 22.5 µl RNase-free water and 1.5 µl cDNA. The reaction mix was vortexed and pulse centrifuged followed by PCR using the following cycle: initial denaturation at 95°C for 2 min; 35 cycles of 3-step cycling: 30 sec denaturation at 95°C, 30 sec annealing at 60°C, 2 min extension at 72°C; final extension 72°C for 5 min, and maintenance at 4°C. To investigate the expression of human *TLRs* in COV434 cells, a commercial primer set was used from Invivogen. The reference genes used for each cell-type (*ACTB* for bovine and human granulosa cells; *L19* for COCs and parthenotes) demonstrated invariant gene expression across all treatments. The qPCR method described in 2.17.1 was MIQE compliant (Bustin et al., 2009).

2.17.1 Quantitative PCR

Semi-quantitative real-time PCR (qPCR) was carried out using the QuantiFast SYBR Green PCR kit (Qiagen), which enables use of primers with different melting points. A master mix was made for each gene containing all components of the kit (per well: 12.5 µl SYBR green, 10.5 µl water, 0.25 µl each primer), except cDNA. Each master mix was vortexed before distributing into wells of a 96-well plate (23.5 µl/well) and 1.5 µl of cDNA added in duplicate or triplicate (for technical replicates; Fig. 2.10). The plate was sealed (Sarstedt) and centrifuged for 3 sec at 3000 x g, before placing in a real-time cycler (BioRad) and using the following program: initial denaturation at 95°C for 5 min followed by 40 cycles of 2-step cycling: 10 sec denaturation at 95°C, 30 sec combined annealing/extension at 60°C; denaturation at 95°C for 1 min; annealing at 55°C for 1 min, and 10 s at each 1°C increase in temperature from 55°C to 95°C to generate a melt curve.

For all plates, a negative control containing the master mix with RNase-free water instead of cDNA was used to verify that the primers were binding specifically and to check whether primer dimers were forming (where the primers bind to each other and this DNA is amplified). The *ACTB* reference gene was used as an internal control for all PCRs using granulosa cell or COV434 cDNA.

In order to determine primer efficiencies, a standard curve was obtained on each plate using a series of 10-fold dilutions of positive control cDNA (from neat cDNA to $1x10^{-3}$ in RNase-free water). If the regression line plotted from the data for the serial dilutions had a coefficient of fit (r^2) of less than 0.97, the experiment was not analysed further (Fig. 2.12). The Ct (Threshold Cycle) values of each set of duplicates or triplicates were examined and any reaction more than 0.2 from another reaction was not analysed. For all PCR reactions, melt curves were also examined (Fig. 2.11). Any samples without a melt curve showing a single smooth peak were not analysed. For each sample, the average starting quantity (calculated by the iCycler software from the standard curve) of the target gene was divided by the average starting quantity of *ACTB* to adjust the quantity of the target gene to the total quantity of cDNA. For some experiments, the fold change in a target gene over a control sample was calculated, by dividing the corrected value for the treated sample by that for the control.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**1**			Sample 1			Sample 5			Sample 9		
B	**0.1**			Sample 1			Sample 5			Sample 9		
C	**0.01**			Sample 2			Sample 6			Sample 10		
D	**0.001**			Sample 2			Sample 6			Sample 10		
E	**1**			Sample 3			Sample 7			Sample 11		
F	**0.1**			Sample 3			Sample 7			Sample 11		
G	**0.01**			Sample 4			Sample 8			Negative Control		
H	**0.001**			Sample 4			Sample 8			Negative Control		

Figure 2.9 A template PCR plate layout for analysis of 2 genes. Triplicate wells contained the reaction for 1 gene shown coloured in purple, and wells containing the reaction for the other gene in white. Master-mix containing no cDNA was used as a negative control.

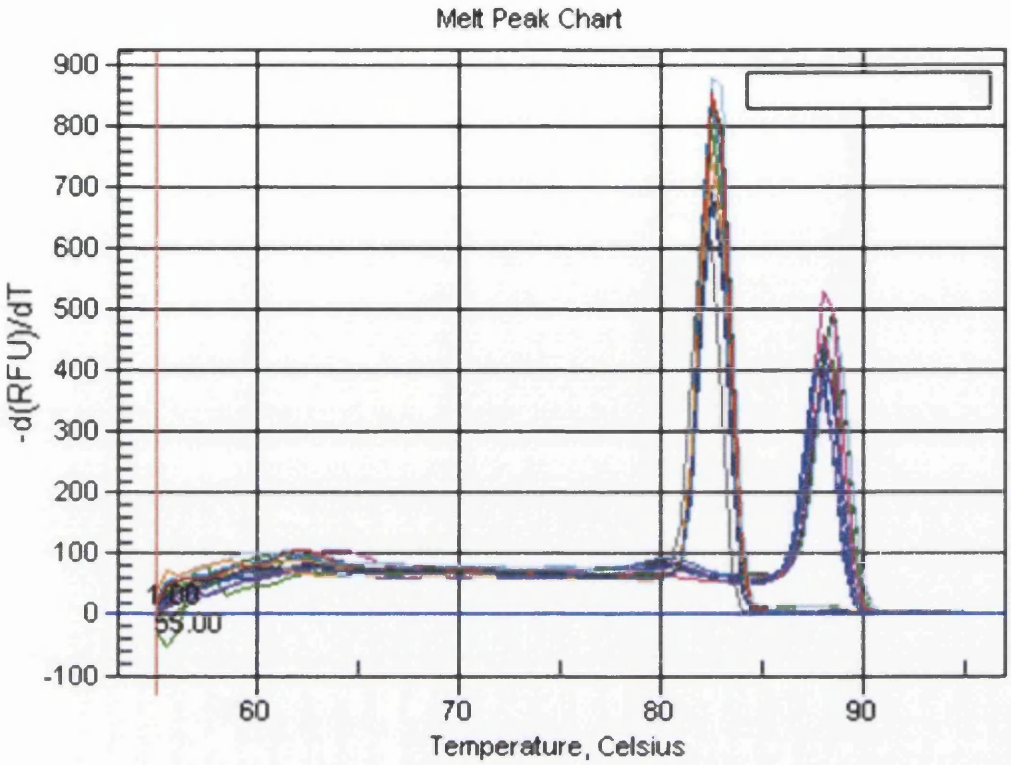


Figure 2.10 Melt curves were examined prior to analysis. A sample melt curve showing the standards for *IL8* (first peak) and *ACTB* (second peak). Genes were analysed one at a time, standard curve first followed by addition of samples. The melt curves were then viewed and any samples without a clean peak were not included in analysis. Triplicates were then examined and any more than 0.2 Ct value different from the other samples were not analysed.

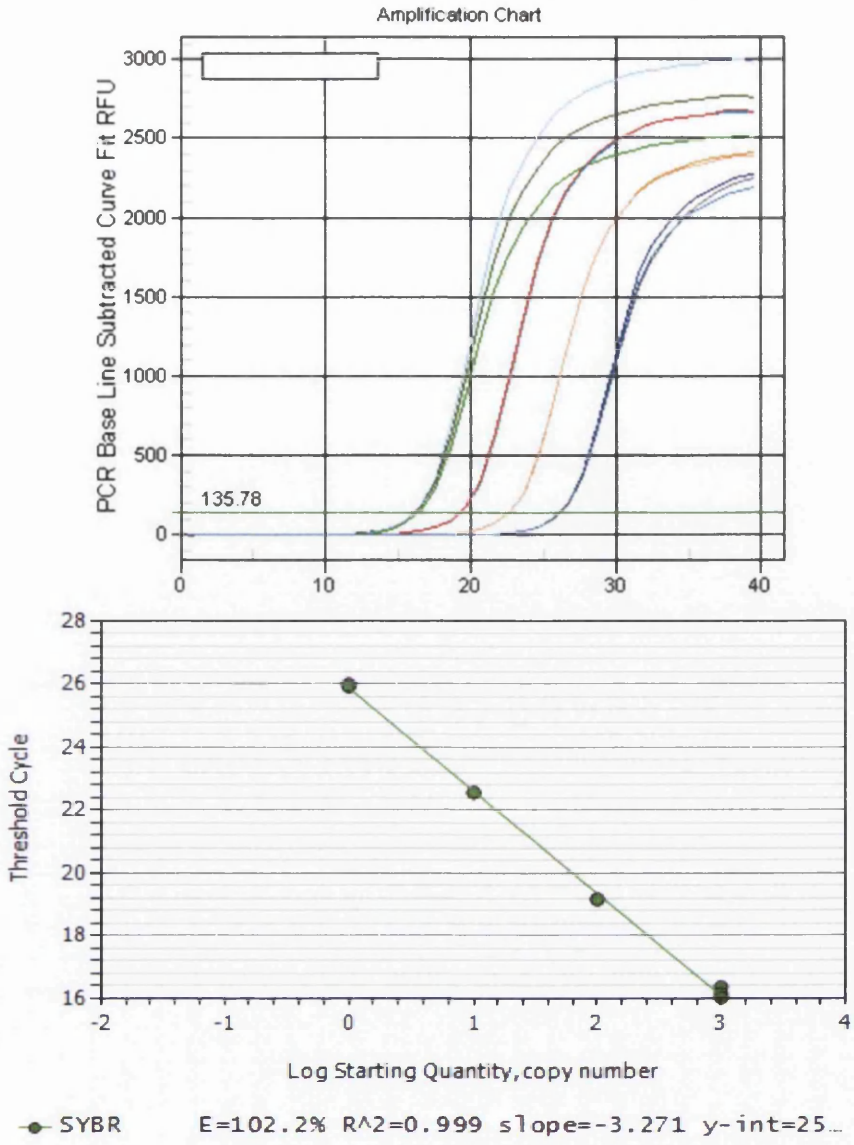


Figure 2.11 A sample qPCR trace for a standard curve. Standards were run in triplicate with the most concentrated standard containing undiluted mRNA and each subsequent dilution being a 1 in 10 dilution of the standard before. A threshold line (bright green) situated on the log-phase of the curve gave the threshold cycle (Ct) value for each standard, enabling a standard curve to be drawn, plotting Ct values against starting concentration of the standards. This resulted in a linear graph with a high degree of correlation (in this case 0.999), enabling the unknown starting quantity of the samples to be estimated from the curve. In addition, primer efficiencies were examined (shown by 'E', in this case 102.2%).

Table 2.2 qPCR primers used in this project including details of GenBank accession number and product length.

Bovine Target	Sense	Antisense	Length	Accession	Reference
ACTB	CAG AAG GAC TCG TAC GTG GG	TTG GCC TTA GGG TTC AGG G	200	NM_173979.3	(Coussens and Nobis, 2002)
IL6	ATG ACT TCT GCT TTC CCT ACC C	GCT GCT TTC ACA CTC ATC ATT C	180	NM_173923.2	(Herath et al., 2009b)
IL1B	CTT CAT TGC CCA GGT TTC TG	CAG GTG TTG GAT GCA GCT CT	142	NM_174093.1	
TNF	CCA TGA GGG CAT TGG CAT AC	CCA TCA ACA GCC CTC TGG TT	138	NM_173966.3	
IL10	TAC TCT GTT GCC TGG TCT TCC T	AGT AAG CTG TGC AGT TGG TCC T	178	NM_174088.1	(Herath et al., 2009b)
IL8	GCA GGT ATT TGT GAA GAG AGC TG	CAC AGA ACA TGA GGC ACT GAA	149	NM_173925.2	
CCL5	TCC ACC CTA GCT CAA CTC CAA	GCC CTG CTG CTT TGC CTA TAT	190	NM_175827.2	
FSHR	AAT CTA CCT GCT GCT CAT AGC CTC	TTT GCC AGT CGA TGG CAT AG	77	NM_174061.1	(Hosoe et al., 2011)
EGFR	ACC ACC CAT CCT GCC TGT ATC AAT	TGC CCA AAC GGA CAA CAT TCT TCC	481	XM_592211.5	(Assidi et al., 2008)
LHCGR	AGA GTG AAC TGA GTG GCT GG	CAA CAC GGC AAT GAG AGT AG	533	NM_174381.1	(Calder et al., 2003)

Bovine Target	Sense	Antisense	Length	Accession	Reference
CYP19A1	GTG TCC GAA GTT GTG CCT ATT	GGA ACC TGC AGT GGG AAA TGA	148	NM_174305.1	
AMH	GTG GTG CTG CTG CTA AAG ATG	TCG GAC AGG CTG ATG AGG AG	104	NM_173890.1	(Rico et al., 2011)
CD45	CTC GAT GTT AAG CGA GAG GAA T	TCT TCA TCT TCC ACG CAG TCT A	185	NM_001206523.1	(Herath et al., 2007)
MHCII	GAG CGG GTG CGG TAC GTG AC	AGC GCC CGG TAC AAG TCC A	86	NM_001034668.3	(Bromfield and Sheldon, 2011)
TLR1	ACT TGG AAT TCC TTC TTC ACG A	GGA AGA CTG AAC ACA TCA TGG A	176	NM_001046504	(Davies et al., 2008)
TLR2	CGG ACA GTC AGC GCA CCA CA	GCT GTC CAC AAA GCA CGT GGC A	196	NM_174197.2	
TLR3	GAT GTA TCA CCC TGC AAA GAC A	TGC ATA TTC AAA CTG CTC TGC T	195	NM_001008664	(Davies et al., 2008)
TLR4	CTTGCGTACAGGTTGTTCCTAA	CTGGGAAGCTGGAGAAGTTATG	153	NM_174198	
TLR5	CCT CCT GCT CAG CTT CAA CTA T	TAT CTG ACT TCC ACC CAG GTC T	172	AY634631	
TLR6	CCT TGT TTT TCA CCC AAA TAG C	TAA GGT TGG TCC TCC AGT GAG T	154	NM_001001159	
TLR7	TCT TGA GGA AAG GGA CTG GTT A	AAG GGG CTT CTC AAG GAA TAT C	205	DQ333225	
TLR8	TAA CCT TCG GAA TGT CTC CAG T	GTG GGA AAT TCT GTT TCG ACT C	232	NM_001033937	

Bovine Target	**Sense**	**Antisense**	**Length**	**Accession**	**Reference**
TLR9	CTG ACA CCT TCA GTC ACC TGA G	TGG TGG TCT TGG TGA TGT AGT C	156	NM_183081	(Davies et al., 2008)
TLR10	ATG GTG CCA TTA TGA ACC CTA C	CAC ATG TCC CTC TGG TGT CTA A	248	NM_001076918	
Human Target	**Sense**	**Antisense**	**Length**	**Accession**	**Reference**
ACTB	ACA GAG CCT CGC CTT TGC CG	TTG CAC ATG CCG GAG CCG TT	108	NM_001101.3	
IL8	GGC CGT GGC TCT CTT GGC AG	TGT GTT GGC GCA GTG TGG TCC	178	NM_000600.3	
IL6	TGT AGC CGC CCC ACA CAG ACA	TGC CAG TGC CTC TTT GCT GCT	145	NM_000576.2	
IL1B	ACG CTC CGG GAC TCA CAG CA	TGA GGC CCA AGG CCA CAG GT	163	NM_000349.2	

2.17.2 Multiplex PCR

For cDNA from COCs and parthenotes, multiplex PCR was used to measure the expression of target genes. Primers and probes were designed using the Eurofins MWG Operon software (conditions: primer length 18 - 22 bp, GC content 40 - 55%, melting temperature 50 - 65°C, PCR product length 100 - 300 bp) and ordered from the same company (Ebersberg, Germany); for sequences see Table 2.3. Primers and probes were reconstituted to 100 µM in TE buffer (Sigma) and stored at -20°C. For each gene, a 20x master mix was made containing 200 µl TE buffer, 20 µl each primer (at 100 µM) and 10 µl corresponding probe (at 100 µM) and stored at -20°C. For each panel of genes, a mastermix was made containing, for each sample, 10 µl multiplex SYBR green (Qiagen), 1 µl each gene mastermix and made up to a total volume of 16 µl with water (scaled up when more than 1 sample). Each reaction was made in duplicate wells of a 96-well low-profile microplate (BioRad) and contained 2 µl cDNA and 8 µl of the panel mastermix. The microplate was sealed (Sarstedt), vortexed briefly and centrifuged for 3 sec at 3000 x g, before placing in a real-time cycler (BioRad) and running the following program: initial denaturation at 95°C for 5 min followed by 50 cycles of 2-step cycling: 15 sec denaturation at 95°C and 30 sec combined annealing/extension at 60°C.

Expression of each target gene was determined relative to *L19* using the comparative CT method (Livak and Schmittgen, 2001), which compares the CT value for the target gene with the CT value for the reference gene (CT value being the point where the fluorescence crosses an arbitrary line determined by the cycler). To calculate the fold change using the ddCT method, the following equations were executed:

1) dCT = CT value for target gene minus CT value for L19 for each sample

2) ddCT = mean dCT value for control samples minus mean dCT value for target

3) fold change = 2 to the power of ddCT

Panel	Target	Sense	Antisense	Probe
1	*PTX3*	CAT CCA ACC ATC TCA TCC AC	ATG CTA AGC CCT GAC TCA C	TCC CTT CAC CCT CTG TCC TCA ACC TTT C Fluorophore: Texas Red; Quench: BHQ2
	HAS2	CAT CCT TTC TTC TGA CTG ACC	CTT CCT TCC ATT CCT AAC CAA C	AAG AAT TGC AGA GCA GAA GCC CCA GGA AG Fluorophore: FAM; Quench: BHQ1
	L19	TAA GAC CAA GGA AGC CTG C	ACA GAG AAG AGG GGG AGA AC	AAA GAG GAA ATC ATC AAG ACT CTG TCC AAG GAG GAA Fluorophore: Cy5; Quench: BBQ
2	*NLPR5*	AGA GTG AGA AGG AAA GAT GAG G	ACA GAC GGG AAA AGG GAA C	TAC AAC GAA ACC TCT CCC GGT GGT Fluorophore: Texas Red; Quench: BHQ2
	GDF9	CCT AAA TCC AAC AGA AGC CAC	TCT TTC TTC TTC CCT CCA CC	TCT ACA ACA CTG TTC GGC TCT TCA CCC CCT Fluorophore: HEX; Quench: BHQ1
	ZAR1	AGT AGA GAG GAA TCG GAC AAG	GCA GAA GGC AGA ACA ACA G	AAT CGA ACT CAC TCA CAG CTC TGC TCTT Fluorophore: FAM; Quench: BHQ1
	L19	As above		As above Fluorophore: Cy5; Quench: BBQ

Panel	Target	Sense	Antisense	Probe
3	*CASP3*	CAC CTC TGC AAA TGA AGC C	CAA AAA AAC CCC CAC AAA CC	ATG GTT TCA TGG TGG TCG GAA GGC TTA Fluorophore: HEX; Quench: BHQ1
	BCL2	CAC CTC CCA CCC AAA TCT AC	TTC CCA ACC CTT CTC AC	CAA ACA ATA AAT GCT GGA GAG GGT GTG G Fluorophore: Texas Red; Quench: BHQ2
	BIRC5	CCT CCC ATT TTT TTC CCC C	CCA AGA CGA CAA ACA ACT CC	GTA GCT TCC ACA ACT TGA ATT TTT TTG CCC Fluorophore: FAM; Quench: BHQ1
	L19	As above		As above Fluorophore: Cy5; Quench: BBQ
4	*GAB1*	ACA CAC ACA CAC ACA CAC CC	CCT TTC CCC AAC ACT TCA ACC	GGA CTG GAT TTT ACA AGT TCT CCC TGA CGA T Fluorophore: HEX; Quench: BHQ1
	CDX2	TGA TAA AGC GGG GAA CGG AG	AGA GAG AGA AAG GTG GTG GG	GTT CCA GAG CCC CAG AGA ACT GGT GTC TAG TGT Fluorophore: Texas Red; Quench: BHQ2
	PLAC8	CCC ACC TAA ATC ACA ACC TCC	GAC AAC TTC CCC GAA ATC AC	GCT CAG GAA TCT ACA TCA TAA ACA ACC TCT CCG AA Fluorophore: FAM; Quench: BHQ1
	L19	As above		As above Fluorophore: Cy5; Quench: BBQ

Panel	Target	Sense	Antisense	Probe
5	*POU5F1*	GAA GTC TTC TGC ATC TAA CCC	AAC CAA TCA CAC CTC CTC C	TCT AGT CTG AGG AAG AGA CAA GTG CAA GCA A Fluorophore: HEX; Quench: BHQ1
	IL6ST	ACA CAC AAA CAC ACA CAC AC	AAG GGA GAC AGA AGA GGA AC	TTC CAA CGC CTT TCT TGA CTC TAC CCC T Fluorophore: Texas Red; Quench: BHQ2
	STAT3	TAA AAC CTC CCC CCT CCA TC	CAA ACT CAC CTC ACC CAA AC	ACT TTC CTA ATC ACT GAA CTT TTC CCT GAC CT Fluorophore: FAM; Quench: BHQ1
	L19	As above		As above Fluorophore: Cy5; Quench: BBQ
6	*DNMT1*	ACA CAC AAA CAC ACA CAC AC	CCC TAA CTC ATA GAC ACT GAC C	ACA ATT TTT GTA GAC TGT TAG GTG GCC TCT Fluorophore: HEX; Quench: BHQ1
	H19	TCC CTC CTT TTC CTC CTT TTC	CAG TGC AAA CGA CTT TTA GTG	CTG ACT CTA TTT TCT GAT CTT CTC TCT GCT ATC CTT TGC T Fluorophore: Texas Red; Quench: BHQ2
	DUSP1	CAG AAA GGG GCA GAA GAG AG	CAG GAA ACA GGG ACA AGA AC	GAG GAC TCA AGT GTG AGA GCC GAT Fluorophore: FAM; Quench: BHQ1
	L19	As above		As above Fluorophore: Cy5; Quench: BBQ

2.17.3 Gel electrophoresis

To visualise PCR products, when required, 2% agarose gels were made by dissolving 2 g molecular grade agarose (Melford, Ipswich, UK) in 10 ml of 10x TAE buffer (0.4 M Tris-base (Melford) + 0.2 M acetic acid (Fisher) + 0.01 M EDTA (Sigma) in H_20) and 90 ml water. This was heated in a conical flask in a microwave at 40% power for about 2 min, until the mix boiled and was clear. When cool to touch, 8 µl ethidium bromide solution (Sigma) was added to the gel mix, the gel was poured into a level mould and a 15-tooth comb was inserted. The gel was cooled at room temperature for around half an hour until set. The comb was then removed and the gel placed in a tank filled with 1x TAE buffer. If BioMix Red was not used, 5 µl of loading buffer (Bio-Rad, Hemel Hempstead, UK) was added to each sample and mixed by pipetting. Then, 12 µl sample (including loading buffer if necessary) was added to each lane on the gel and 5 µl of a 100 base pair ladder (Bio-Rad) loaded at each end of the samples. Electrophoresis was started at 120 V for 5 min and then continued at a constant 85 V (PowerPac Basic, Biorad). An image of the gel was taken using the GelDoc 2000 system (Bio-Rad) and the associated Quantity One Software (Bio-Rad).

2.18 Protein extraction and quantification

Granulosa cells were washed once using room-temperature PBS before being lysed using 80 µl Phosphosafe Extraction Reagent (Novagen, Madison, US) per well of a 12-well plate (TPP). Adherent cells were removed from each well using a sterile cell scraper (Greiner Bio-One) and the lysate transferred to 0.5 ml tubes (Eppendorf). These tubes were then centrifuged at 21,000 x g at 4°C for 5 min before quantification of protein and storage at -20°C. A detergent compatible (DC) assay (Biorad) was used to quantify the protein concentration of each sample according to the manufacturer's instructions. The DC assay is similar to the Lowry assay but requires only 15 min incubation time instead of 40 min (Lowry et al., 1951). Addition of an alkaline copper solution to the protein enables the protein to reduce Folin Reagent by loss of 1 to 3 oxygen atoms, producing a blue colour with a maximum absorbance at 750 nm. The development of colour is mainly due to oxidation of tyrosine and tryptophan, but also cystine, cysteine and histadine. The greater the absorbance at 750 nm, the greater the reduction of Folin Reagent, thus the greater the starting quantity of protein.

Briefly, bovine serum albumin (Sigma) was diluted in water to generate standards from 0.5 to 2.5 mg/ml. Standards and samples were tested in duplicate, made directly in a 96-well plate (TPP) to a total volume of 5 µl/well. Then, 20 µl of reagent S (Biorad) was added to each 1 ml of reagent A, an alkaline copper tartrate solution (Biorad; forming reagent A') needed for the assay and mixed by vortexing. Twenty-five µl of reagent A' was added into each well containing sample or standard, followed by 200 µl reagent B, a dilute Folin reagent (Biorad). The plate was incubated at room temperature for 15 min, before reading absorbance at 750 nm using a microplate reader (POLARstar Omega, BMG Labtech, Offenburg, Germany). The standards were used to create a linear standard curve by plotting absorbance at 750 nm against protein concentration and the concentration of samples was determined using this standard curve ***(y = mx+c)***.

2.18.1 SDS-PAGE

For sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), 10 μg each protein sample was mixed with an equal volume of Laemmli buffer (Sigma) in 0.2 ml thin-walled tubes (Eppendorf), vortexed, pulse centrifuged and heated for 5 min at 95°C. Mini PROTEAN TGX 12% precast polyacrylamide gels were assembled in a mini trans-blot PROTEAN electrophoresis cell (both Biorad). The tank was filled with running buffer (25 mM Tris (Melford), 192 mM glycine (Melford), 0.1% w/v SDS (Invitrogen) in water) and surrounded with ice. Then, 5 μl Precision Plus Protein Kaleidoscope standards (Biorad) was added to the first and last well, and samples were added to the remaining wells. Electrophoresis was carried out at 200 V for 45 min (PowerPac Basic, Biorad).

2.18.2 Western blot

Polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Chalfont St Giles, UK) was immersed in 100% methanol (Fisher) until translucent. Fibre pads and blot paper (both Biorad) were equilibrated for 10 min in transfer buffer (20 mM Tris, 192 mM glycine, 20% v/v methanol, 0.1% w/v SDS), cooled to 4°C. The transfer cassette was assembled as shown in Fig 2.12, surrounded by ice and protein was transferred at 400 mA for 1 h at 4°C. The membrane was then removed from the cassette and blocked in 5% BSA (Sigma) in transfer buffer overnight at 4°C with gentle agitation. The membrane was then incubated with primary antibody (see Table. 2.4) at room temperature for 1 h 30, before washing 4 x 5 min with TBS/T (20 mM Tris, 125 mM NaCl, 0.1% v/v Tween20 (all Sigma) in water). The membrane was incubated for 1 h with secondary antibody at room temperature, followed by a further 4 x 5 min washes with TBS/T, before placing the membrane on the stage of the ChemiDoc XRS system (Biorad). Then, 1 ml Luminata Forte Western HRP substrate solution (Millipore, Billerica, USA) was used to cover the membrane and an image was collected, using Quantity-One software (Biorad) before saturation occurred (using the highlight saturated pixel function of the program). In order to probe for β-actin, the membrane was then incubated in Restore Western Blot Stripping Buffer (Thermoscientific, Erembodegem, Belgium) at room temperature for 7 min, reblocked and restained with the appropriate primary and secondary antibodies.

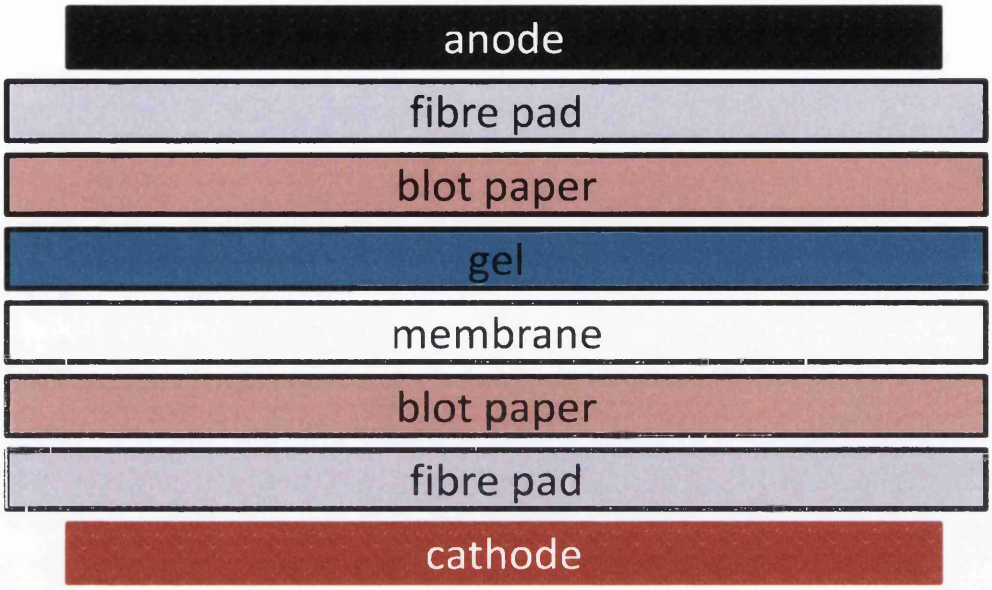


Figure 2.12 The transfer cassette for protein transfer from the gel to the membrane. PVDF membrane was immersed in 100% methanol until translucent and fibre pads and blot paper (all Biorad) were equilibrated for 10 min in transfer buffer (20 mM Tris, 192 mM glycine, 20% v/v methanol, 0.1% w/v SDS) pre-cooled to 4°C. The transfer cassette was assembled surrounded by ice and the protein was transferred at 400 mA for 1 h at 4°C.

Table 2.4 Antibodies used for Western blot analysis.

Target peptide or protein	Band Size	Manufacturer and code	Dilution	Species raised in and clonality
diphosphorylated ERK1/2	42/44 kDa	Sigma M8159	1:1000	mouse monoclonal
p38 MAPK (pThr180/pTyo182)	39 kDa	Acris Antibodies AP05898PU-N	1:1000	rabbit polyclonal
beta-actin	42 kDa	Abcam ab8226	1:1000	mouse monoclonal
mouse IgG-HRP linked	N/A	Cell Signalling 7076	1:1000	horse polyclonal
rabbit IgG-HRP linked	N/A	Cell Signalling 7074	1:1000	goat polyclonal

2.18.3 Quantification of Western blots

Images of Western blots were analysed using the Quantity-One software (BioRad), as shown in Fig. 2.13. Firstly, lanes were framed and the bands detected by the software. A rolling background was then set to compensate for any changes in background intensity. The peak density of each band was measured by the software. For p-ERK1/2, the peak density of p-ERK1 and p-ERK2 were added together. In order to correct for total protein loading, the peak density of bands representing either p-p38 MAPK or p-ERK1/2 was divided by the peak density of the band representing β-actin. These figures were then divided by the ratio for time 0 to give a percentage increase in phosphorylation of p38 MAPK or ERK1/2 over time.

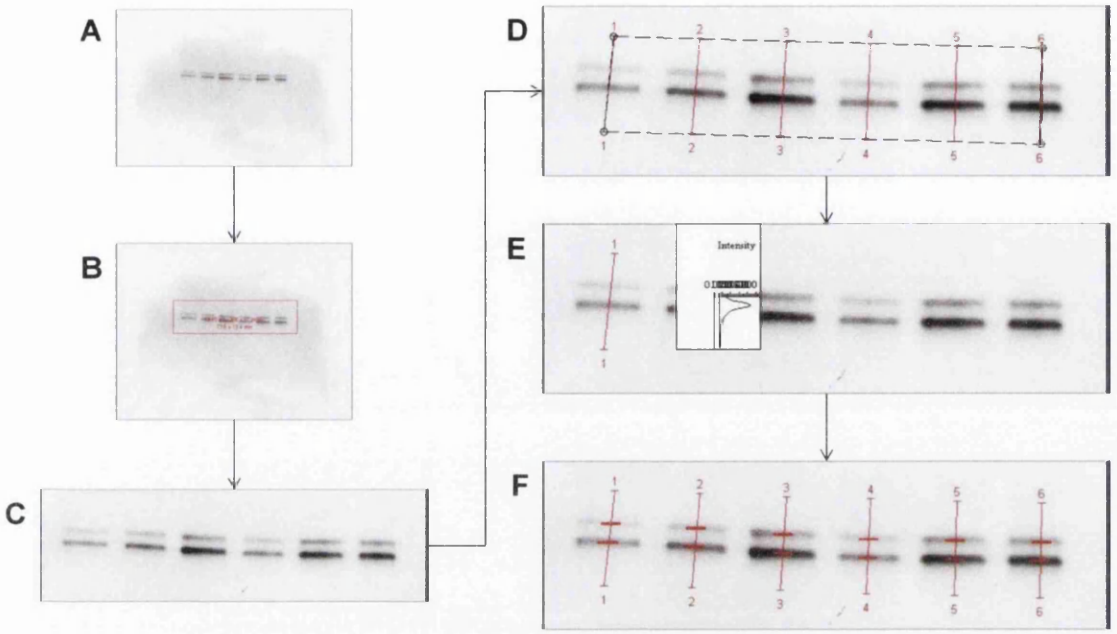


Figure 2.13 Western blots were analysed using the Quantity One software (BioRad). The raw image (A) was cropped to include only the area of interest (B). The contrast of the image was then adjusted to optimise the background (C). Using the program's software, lanes were framed, so that the centre of each lane bisected each set of bands in the middle (D). The angle of each lane was adjusted as necessary to run parallel with the blot. The background was then adjusted to allow for any changes in background intensity using the rolling background function (E). Lastly, bands were detected (F) and the peak density measured.

2.19 Enzyme-linked immunosorbent assay

The accumulation of inflammatory mediator protein in culture supernatants was examined using enzyme-linked immunosorbent assay (ELISA; Table 2.5). Samples from human cells were examined using ELISAs specific for human inflammatory mediators. Samples from bovine cells were examined using ELISAs specific for bovine inflammatory mediators, except for IL-8. The human IL-8 ELISA was used for bovine samples as it has previously been shown to cross-react with bovine IL-8 (Shuster et al., 1997).

Table 2.5 Assays used to measure accumulation of protein in cell supernatants

Target	Name	Manufacturer	Code
hIL-6	Human IL-6 DuoSet	R&D Systems, Abingdon, UK	DY206
hIL-1β	Human IL-1 beta/IL-1F2 DuoSet		DY201
hIL-8	Human CXCL8/IL-8 DuoSet		DY208
hCCL5	Human CCL5/RANTES DuoSet		DY278
bTNFα	Bovine TNF-alpha DuoSet		DY2279
bIL-6	Bovine IL-6 ELISA Reagent Kit	Thermo-Scientific, Rockford, USA	ESS0029
bIL-1β	Bovine IL-1 beta ELISA Reagent Kit		ESS0027
bIL-8 (COC)	In-house		
E2 (COV434)	In-house (Walker et al., 2002, Atsalis et al., 2004, Lueders et al., 2009, Pollock et al., 2010)		
E2	Oestradiol ELISA	DRG Diagnostics, Marburg, Germany	EIA-2693
P4	Progesterone ELISA		EIA-1561

2.19.1 R&D Systems cytokine/chemokine ELISA

Details of reagents and buffers can be found in Tables 2.6 and 2.7. The limit of detection, inter- and intra-assay variability can be found in Table 2.9. Capture antibody was diluted to working concentration in PBS and used to coat a half-area 96-well microplate (Greiner Bio-One) and the plate was sealed and incubated at 4°C overnight on a shaker. Each well was then aspirated and washed 3 times with wash buffer (140 µl/well). After the final wash, the plate was inverted and blotted against clean paper towels. Then, 150 µl of block buffer was added to each well and incubated for 1 h on a shaker, followed by a further wash, as described above. The most concentrated standard was made in reagent diluent followed by 2-fold serial dilutions in reagent diluent. Supernatants were defrosted overnight at 4°C and vortexed before use. Then, 50 µl of standard or supernatant was added to each well in duplicate and the plate was incubated at room temperature for 2 h. The plate was then washed 3 times and 50 µl of detection antibody diluted to working concentration in reagent diluent was added to each well, followed by incubation at room temperature for 2 h. After a further 3 washes, streptavidin conjugated to horseradish peroxidase (streptavidin-HRP; R&D Systems) was diluted in reagent diluent (30 µl in 6 ml) and 50 µl was added to each well. The plate was then incubated in the dark for 20 minutes at room temperature. A final wash step preceded addition of 50 µl substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine; BD Biosciences) per well, incubated for 20 minutes at room temperature in the dark. Finally, 50 µl of stop solution (2N H_2SO_4) was added to each well and the plate was analysed.

A spectrophotometer (POLARstar Omega) and associated software (MARS data analysis v1.2 R2, Omega) was used to determine the concentrations of cytokines. The software was used to plot optical density against the concentration of the standard (pg/ml). A standard curve was formed using the 4-parameter fit model based on the blank corrected raw data and the software calculated the unknown concentrations of the samples from this standard curve (for equation see Fig. 2.14). Data were analysed only if the coefficient of fit of the curve was greater than 0.990.

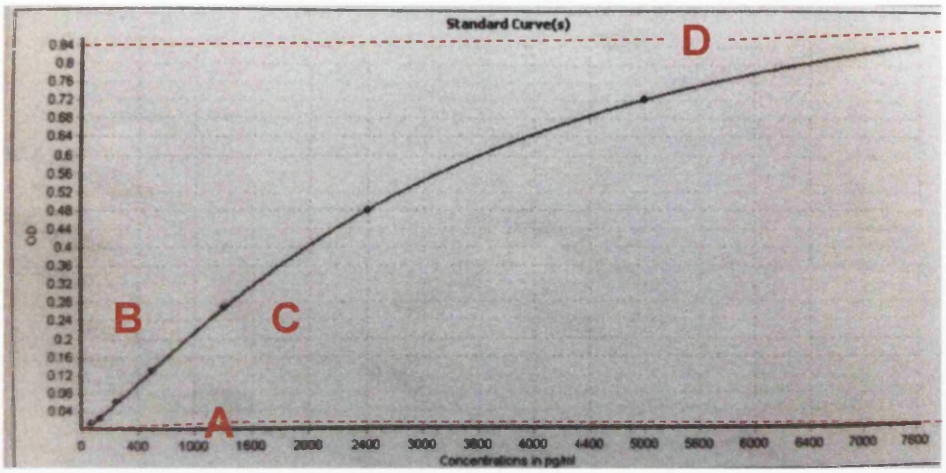


Figure 2.14 ELISA standards were fitted to a 4-parameter curve. A 4-parameter fit works using the equations $y = ((A-D) / (1 + (x/C)^B)) + D$, where A represents the response at 0 pg/ml, B represents the gradient, C represents the inflection point, where the curvature changes direction and D represents the response at an infinite concentration.

Table 2.6 Solutions used for each ELISA (all reagents from Sigma unless otherwise stated). Wash buffer, block buffer and reagent diluents were made in advance and stored for up to 2 weeks at 4°C; substrate solution was made immediately before use and stop solution was made in advance and kept at room temperature for up to 6 weeks

	IL-8	**IL-6**	**IL-1β**	**TNFα**
PBS	4 PBS tablets dissolved in 1 L water			
Wash Buffer	0.05% Tween 20 in PBS, pH7.2-7.4			
Block Buffer	1% BSA in PBS	Same as Reagent Diluent		
Reagent Diluent (RD)	0.1% BSA, 0.05% Tween 20 in TBS (20mM Trizma base, 150 mM NaCl) pH 7.2 - 7.4 0.2 μM filtered	1% BSA in PBS pH 7.2 - 7.4 0.2 μM filtered		5% Tween 20 in PBS 0.2 μM filtered
Substrate Solution	1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (tetramethylbenzidine) (R&D Systems)			
Stop solution	2N Sulphuric Acid Solution			

Table 2.7 Reagents (including working concentration) used for each ELISA (all from R&D Systems, Abingdon, UK). Capture antibody was reconstituted in PBS; detection antibody was reconstituted in reagent diluents. Both were divided into aliquots and stored at -20°C for up to 6 months. Recombinant protein standards were reconstituted with deionised water, divided into aliquots and stored at -20°C

	IL-8	**IL-6**	**IL-1β**	**TNFα**
Capture Antibody	Mouse anti-human IL-8	Mouse anti-human IL-6	Mouse anti-human IL-1B	Goat anti-bovine TNFα
Part Number	890804	840113	840168	842628
Working Conc.	4.0 µg/ml	2.0 µg/ml	4.0 µg/ml	0.8 µg/ml
Detection Antibody	Biotinylated goat anti-human IL-8	Biotinylated goat anti-human IL-6	Biotinylated goat anti-human IL-1B	Biotinylated goat anti-bovine TNFα
Part Number	890805	840114	840169	842629
Working Conc.	20 ng/ml	200 ng/ml	300 ng/ml	1200 ng/ml
Standard	Recombinant human IL-8	Recombinant human IL-6	Recombinant human IL-1B	Recombinant bovine TNFα
Part Number	890806	840115	840170	842630
Top concentration	2000 pg/ml	600 pg/ml	250 pg/ml	8000 pg/ml
Streptavidin-HRP	Part number 890803 Diluted 1:200			

2.19.2 Thermo-Scientific cytokine ELISA

Wash buffer (0.05% Tween20 in PBS), blocking buffer (4% BSA, 5% sucrose in PBS (all Sigma); 0.2 μM filtered), carbonate-bicarbonate buffer (0.2 M made using carbonate-bicarbonate buffer capsules; Sigma) and reagent diluent (4% BSA in PBS; 0.2 μM filtered) were made in advance and stored for up to 2 weeks at 4°C. The limit of detection, inter- and intra-assay variability can be found in Table 2.8.

Coating antibody was diluted 1:100 in carbonate-bicarbonate buffer and used to coat a half-area 96-well microplate (50 μl/well). The plate was sealed and incubated at 4°C overnight on a shaker. Each well was aspirated and 150 μl of block buffer was added to each well and incubated for 1 h on a shaker. The plate was then washed 3 times using a plate washer (LT-3500, Labtech, Ringmer, UK). The provided standard was reconstituted in reagent diluent, and reagent diluent was used to prepare 1:2 serial dilutions (highest standard concentration for IL-6 was 5000 pg/ml; for IL-1β it was 2000 pg/ml). Supernatants were defrosted overnight at 4°C and vortexed before use. Then, 50 μl of standard or supernatant was added to each well in duplicate and the plate was incubated at room temperature for 1 h. The plate was then washed 3 times and 50 μl of detection antibody diluted 1:100 in reagent diluent was added to each well, followed by incubation at room temperature for 1 h. After a further 3 washes, streptavidin-HRP was diluted 1:400 in reagent diluent and 50 μl added to each well. The plate was then incubated in the dark for 30 minutes at room temperature. A final wash step preceded addition of 50 μl substrate solution per well, which was incubated for 20 minutes at room temperature in the dark. Finally, 50 μl of stop solution was added to each well and the plate was analysed using a microplate spectrophotometer and associated software, as described above.

2.19.3 IL-8 ELISA for COCs

An ultrasensitive IL-8 ELISA was developed in house, ideal for supernatants from a small number of cells, for example COCs. Wash buffer (0.05% Tween20 in PBS), carbonate-bicarbonate buffer (0.2 M made using carbonate-bicarbonate buffer capsules) and reagent diluent (4% fish-skin gelatine (Sigma) in PBS; 0.2 μM filtered) were made in advance and stored for up to 2 weeks at 4°C. The limit of detection, inter- and intra-assay variability can be found in Table 2.9.

Coating antibody (mouse anti-sheep IL-8; MCA1660; AbD Serotec) was diluted 1:400 in carbonate-bicarbonate buffer and used to coat a half-area 96-well microplate (50 μl/well). The plate was sealed and incubated at room temperature overnight on a shaker. Each well was aspirated, washed 3 times using a plate washer and 150 μl of reagent diluent was added to each well for 1 h. The plate was then washed 3 times using a plate washer and standards (bovine IL-8, RP0023B-025; Kingfisher Biotech, St Paul, USA) prepared in reagent diluent using 1:2 serial dilutions (highest standard 4000 pg/ml). Supernatants were defrosted overnight at 4°C and vortexed before use. Then, 50 μl of standard or supernatant was added to each well in duplicate and the plate was incubated at room temperature for 90 min. The plate was then washed 3 times and 50 μl of detection antibody (rabbit anti-sheep IL-8; AHP425; Abd Serotec) diluted 1:700 in reagent diluent was added to each well, followed by incubation at room temperature for 2 h. After a further 3 washes, the tertiary antibody (HRP-conjugated goat anti-rabbit; P0448; Dako, Glostrup, Denmark) was diluted 1:6000 in reagent diluent and 50 μl added to each well and incubated for 1 h at room temperature. After a final wash step, 50 μl substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) was added to each well, and incubated for 10 minutes at room temperature in the dark. Finally, 50 μl of stop solution (2N sulphuric acid) was added to each well and the plate was analysed using a microplate spectrophotometer and associated software, as described above.

2.19.4 Oestradiol ELISA for COV434 cells

This oestradiol ELISA was only used for supernatant from COV434 cells (chapter 6). All buffers were made in advance and stored at 4°C for up to 3 weeks (for formulations see Table 2.8). The limit of detection, inter- and intra-assay variability can be found in Table 2.9. The oestradiol capture antibody was diluted 1:100 in coating buffer and stored in aliquots at -20°C. Oestradiol-17β-horseradish peroxidise (E2-HRP) was diluted 1:500 in EIA buffer and stored at 4°C and used at a 1:50,000 dilution in EIA buffer. Ethanol was used to dilute 17β-oestradiol (Sigma) to a concentration of 10 mg/ml and stored at -20°C. This was then diluted further in EIA buffer to give a working stock of 100 ng/ml. Polyclonal anti-oestradiol R4972 (a kind gift from Jean Routley, University of Liverpool) cross-reacts with oestradiol-17β (100%), oestrone (3.3%), androstenedione (1%), progesterone (0.8%) and less than 1% with cortisone and dihydrotestosterone (Gudermuth et al., 1998).

The antibody was diluted from stock concentration of 1:100 to a working dilution of 1:10,000 using coating buffer (i.e. 50 µl antibody stock in 5 ml coating buffer) and used to coat a half-area 96-well microplate. The plate was sealed and incubated at 4°C overnight on a shaker. Each well was then aspirated and washed 5 times with wash buffer (140 µl/well). After the final wash, the plate was inverted and blotted against clean paper towels. Immediately, 50 µl of EIA assay buffer was added to each well and incubated for 2 h at room temperature on a shaker. The most concentrated standard (25 ng/ml) was made in EIA buffer followed by 2-fold serial dilutions in EIA buffer. Supernatants were defrosted overnight at 4°C and vortexed before use. Without washing the plate, 20 µl of standard or supernatant and 50 µl of E2-HRP was added to each well. The plate was then incubated at room temperature for 2 h. The plate was washed 5 times as above. The ABTS substrate was prepared immediately before use and 100 µl was added to each well. The plate was incubated at room temperature in the dark to allow colour to develop (30-60 min) and the optical density determined immediately using a microplate reader set to 405nm.

Table 2.8 Oestradiol ELISA buffers: all products from Sigma unless otherwise stated.

Coating Buffer pH 9.6	1.59 g Na_2CO_3 (Anhydrous) 2.93 g $NaHCO_3$ 1 l water
EIA buffer pH 7.0	Stock A: 27.8 g 0.2 M NaH_2PO_4 in 1 l water Stock B: 28.4 g 0.2 M Na_2HPO_4 in 1 l water 195 ml Stock A 305 ml Stock B 8.7 g NaCl 1 g BSA 500 ml water
Wash concentrate	87.66 g NaCl 5 ml Tween 20 1 l water (dilute 1 in 10 in water for use)
Substrate buffer	9.61 g Citric acid (anhydrous) 1 l water
ABTS pH 6.0 Keep in dark	ABTS: 0.55 g ABTS in 25 ml water H_2O_2: 500 µl hydrogen peroxide in 8 ml water 125 µl 40 mM ABTS 125 µl 0.5 M hydrogen peroxide 12.5 ml substrate buffer

2.19.5 Oestradiol and Progesterone ELISA (DRG Diagnostics)

Due to limitations using the in-house oestradiol ELISA, associated with high inter-assay variability and limit of detection, the accumulation of oestradiol and progesterone in supernatant from bovine GCs was measured using commercial kits (DRG Diagnostics, Marburg, Germany). These commercial kits are based on competitive-binding and use ELISA wells pre-coated with an antibody specific to the target. All reagents were warmed to room temperature before use and were used according to the manufacturer's instructions.

Briefly, to measure the accumulation of oestradiol, 25 µl of standard (0, 25, 100, 250, 500, 1000 or 2000 pg/ml) or sample was pipetted into duplicate wells. This was immediately followed by addition of 200 µl/well of enzyme conjugate (oestradiol conjugated to horse-radish peroxidase). The contents were mixed by repeat pipetting for 10 sec, before incubating for 2 h at room temperature. Then, the contents of the wells were expelled, wells were washed 3 times (400 µl/well) and residual liquid expelled by forcefully blotting the wells on tissue paper. Then, 100 µl/well substrate solution (tetramethylbenzidine) was incubated for 15 min at room temperature. Finally, 50 µl/well stop solution (0.5 M sulphuric acid) was used to prevent further colour development and the plate was read at 450 nm (Omega, as above).

To measure the accumulation of progesterone, 25 µl of standard (0, 0.3, 1.25, 2.5, 5, 15 or 40 pg/ml) or sample was pipetted into duplicate wells and incubated for 5 min. Then 200 µl/well of enzyme conjugate (progesterone conjugated to horse-radish peroxidase) was added and mixed by repeat pipetting for 10 sec, before incubating for 1 h at room temperature. The plates were washed as above and 200 µl/well substrate solution (tetramethylbenzidine) was incubated for 15 min at room temperature. Finally, 50 µl/well stop solution (0.5 M sulphuric acid) was used to prevent further colour development and the plate was read at 450 nm (Omega, as above).

Table 2.9 Limit of detection, inter- and intra- assay variability for ELISAs used in this project. The inter-assay variability was calculated by dividing the standard deviation by the mean of the same sample run on 4 different plates (x100). The intra-assay variability was calculated by dividing the standard deviation by the mean of the same sample run on the same plate in multiple wells (x100). The limit of detection was calculated by adding 3 times the standard deviation of the lowest standard (over 4 plates) to the blank.

ELISA	**Limit of Detection pg/ml**	**Intra-assay variability %**	**Inter-assay variability %**
hIL-6	8.2	1.2	2.4
hIL-1β	5.1	4.6	7.7
hIL-8	14.3	1.7	5.5
hCCL5	10.6	4.5	4.9
bIL-6	35.6	1.2	3.0
bIL-1β	20.1	4.6	7.7
bTNFα	21.8	3.1	3.8
bIL-8 (in house)	47.3	2.2	2.7
In house E2	333.5	13.0	21.2
E2 (DRG)	10.7	1.1	1.2
P4 (DRG)	40	5.0	3.8

2.20 MTT assay

Cell survival was estimated using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). A stock solution of 5 mg/ml MTT (Sigma) in PBS was used at a working concentration of 0.5 mg/ml in culture medium. After removal of the cell supernatants, cells grown in a 24-well plate were washed in 350 µl PBS and 250 µl of the MTT at 0.5 mg/ml was added to each well, followed by incubation at 37°C for 1 h. The cells were then washed in 350 µl PBS and 250 µl of demethyl sulphoxide (DMSO; Sigma) was added to each well to lyse the cells. The plate was placed on a rocker for 15 mins and then 50 µl lysed cell suspension was pipetted into a 96-well plate (TPP) in duplicate. The plate was analysed using a spectrophotometer (POLARstar Omega) at 570 nm and associated software (MARS data analysis v1.2 R2, Omega). Results are expressed as a percentage of the absorbance for the supernatant from cells cultured in control medium.

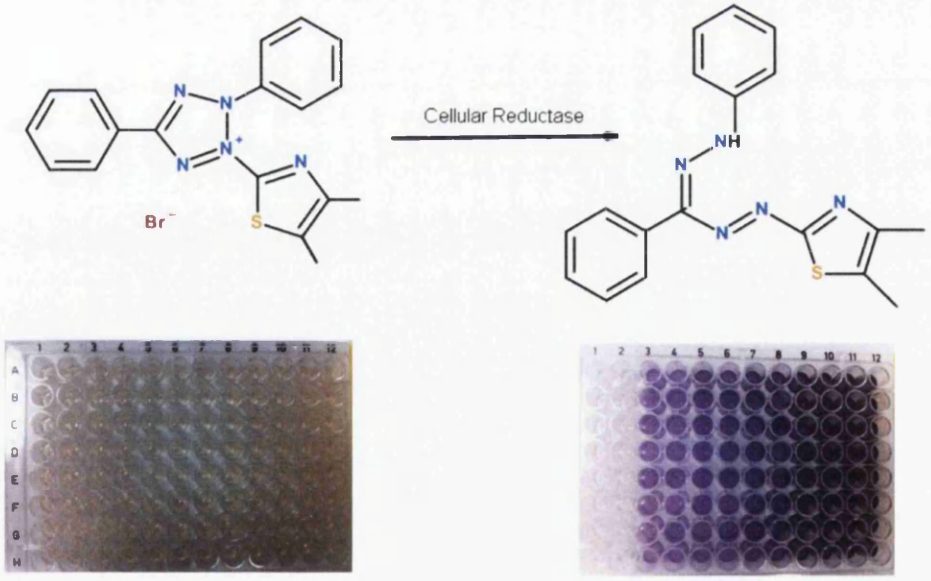


Figure 2.15 The MTT estimated cell viability. MTT is yellow in its bromide state but the positively charged tetrazolium ring is cleaved by active mitochondrial hydrogenases to purple formazan. The cells are then lysed using DMSO, and the absorbance measured using a microplate reader.

2.21 ApoTox Glo assay

To further investigate the effect of treatments on cell health, an ApoTox-Glo Triplex Assay was used, according to the manufacturer's instructions (Fig. 2.16; Promega). Prior to starting the assay, the reagents were made and stored at 4°C. To make the viability/cytotoxicity reagent, 10 µl of GF-AFC substrate and 10 µl bis-AAF-R110 substrate were mixed by vortexing in 2 ml of assay buffer. To make the Caspase-Glo 3/7 reagent, the entire Caspase-Glo 3/7 buffer was mixed by inversion with the Caspase-Glo 3/7 substrate, in the bottle containing the substrate.

After cells had been treated in duplicate in a 96-well plate, supernatants were removed and 100 µl culture medium added to each well. Then, 20 µl viability/cytotoxicity reagent was added to each well, mixed by orbital shaking (300 rpm for 30 s) and incubated for 30 min at 37°C. Fluorescence was then measured with excitation at 400 nm and emission at 505 nm to assess cell viability, and 485 nm excitation and 520 nm emission to assess cytotoxicity. Then, 100 µl Caspase-Glo 3/7 reagent was added to each well, mixed by orbital shaking (300 rpm for 30 s) and incubated for 30 min at room temperature. Luminescence was then measured to assess caspase activation, a marker of apoptosis.

Two positive controls were used in this assay: 15 min digitonin (Sigma) treatment at 30 µg/ml was used as a positive control for cytotoxicity, and 10 µM staurosporine (Sigma) treatment for 4 h was used as a positive control for apoptosis and viability.

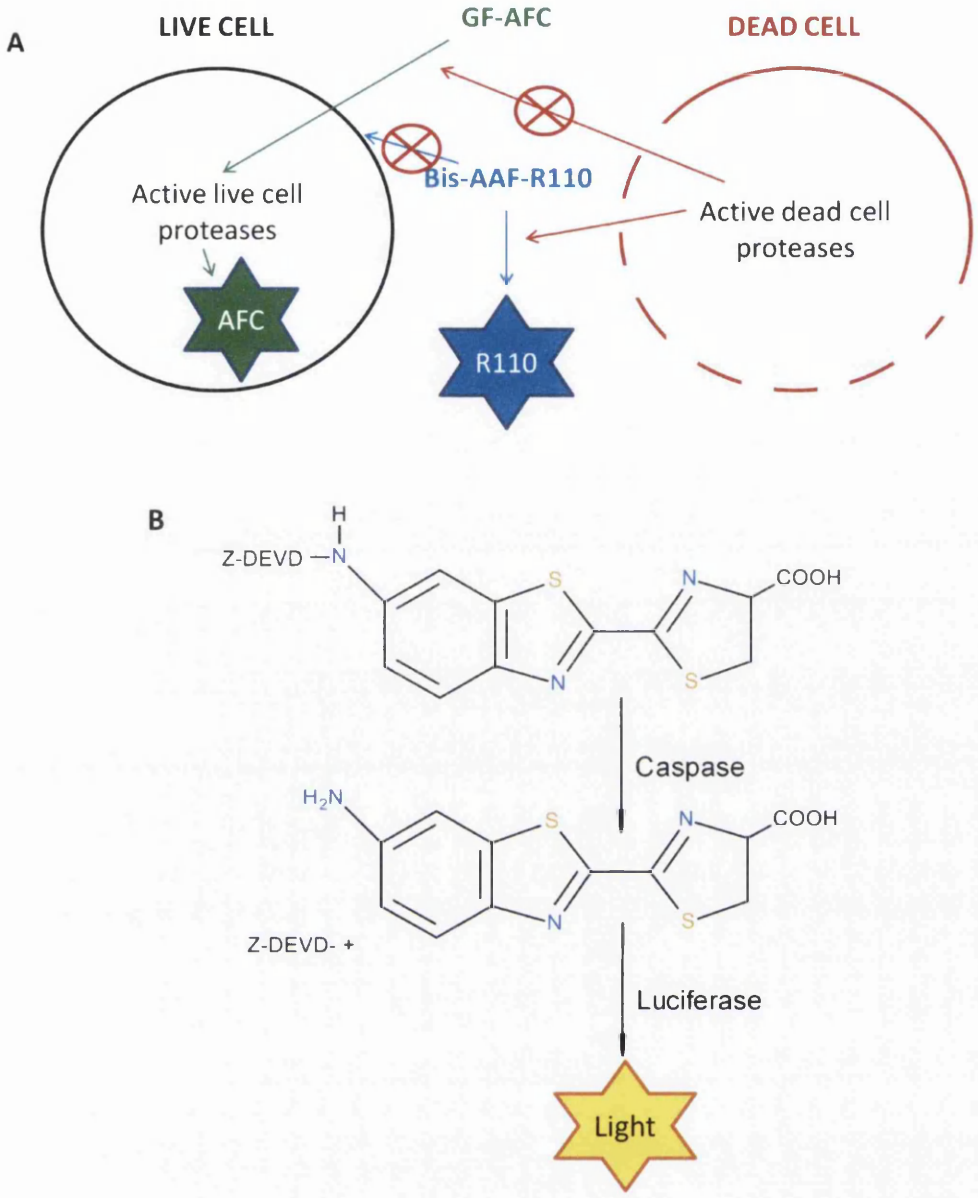


Figure 2.16 The ApoTox Glo Triplex Assay (Promega) evaluated cell viability, toxicity and apoptosis. In step A, 2 proteins are added to the cells; one measures cell viability and the other measures cytotoxicity. GF-AFC is cell permeable and is cleaved by live cell proteases to AFC, which is fluorogenic with excitation of 400 nm and emission of 505nm. Dead cells to not have active live cell proteases thus AFC is not formed. Bis-AAF-R110 is not cell permeable, thus cannot enter live cells. However, dying cells without an intact cell membrane leak dead cell proteases, which cleave Bis-AAF-R110 to R110, which fluoresces with excitation of 485 nm and emission of 520 nm. In step B, cells are lysed and caspases cleave the substrate to produce light through luciferase action.

2.22 Statistical analyses

Statistical analyses were performed using SPSS version 19 (SPSS Inc) and significance ascribed when $P < 0.05$.

Data were examined for normality by drawing a histogram of the data. For normally-distributed data, following square root or log transformation where indicated in *Results*, either Student's T-test or analysis of variance (ANOVA) was used to test for significant differences between groups. For data within 2 groups, Student's T-test was used to compare the two groups. The T-test examines differences between the mean of 2 groups of data, comparing the means of each group with the variation in each group. For normally-distributed data with more than 2 groups, ANOVA was used, with Dunnett's pairwise multiple comparison T-test used to compare treatments with control. The post-hoc Dunnett's T-test function runs multiple T-tests comparing treatments against a selected control, but is more conservative than running multiple Student's T-tests, as it references against a different table. When the aim was to compare each group with every other group in an experiment (rather than compare against control), Fisher's least significant difference post-hoc test was used.

When data were not normally distributed, comparisons between treatment and control were made using Mann-Whitney U test, as indicated in *Results*. For discontinuous results, for example the grading of cumulus expansion, groups were compared using Fisher's exact test.

Chapter 3

Granulosa cells from emerged antral follicles of the bovine ovary initiated inflammation in response to bacterial pathogen-associated molecular patterns via Toll-like receptor pathways

3.1 Introduction

Bacterial contamination of the uterus is ubiquitous after parturition in cattle (Sheldon et al., 2002) with 40% of animals developing clinical disease (Sheldon et al., 2009). In addition to causing distress and suffering to the animal, uterine disease causes infertility, not only through endometrial damage, but also by perturbation of ovarian function. Cows with uterine disease exhibit slower growth of dominant follicles and have lower peripheral plasma oestradiol concentrations than healthy animals (Sheldon et al., 2002). However, it is not clear if uterine disease impacts emerged ovarian follicles.

In cattle, follicles develop in follicular waves, with a cohort of 6 to 24 ovarian follicles synchronously emerging in response to a rise in peripheral plasma FSH concentrations (Ginther et al., 1996). The concentration of FSH begins to decline after a further 2 days and only larger follicles with more FSH receptors are able to survive, whilst remaining follicles undergo atresia. As FSH concentrations continue to fall, one follicle is selected as the dominant follicle, becoming responsive to luteinising hormone (LH). Granulosa cells (GCs) are somatic cells which line ovarian follicles and support the growth and development of the oocyte. At follicle emergence, GCs begin to express the FSH receptor, and develop the receptor for LH when a follicle becomes dominant. Thus, follicles are dependent on GCs to grow and develop. This chapter focused on the impact of uterine disease on GCs from emerged follicles.

Uterine disease is associated with both Gram negative and Gram positive bacteria, including *E. coli* and *T. pyogenes* (Williams et al., 2005). *Escherichia coli* is the first bacterium to invade the endometrium of the post-partum cow, paving the way for infection by *T. pyogenes* (Williams et al., 2007). *Trueperella pyogenes* infection is associated with increased severity of uterine lesions and acts synergistically with other bacteria to cause tissue damage and severe endometritis (Bonnett et al., 1991).

Escherichia coli is a Gram negative, facultive anaerobic bacterium, part of the normal flora of the gastrointestinal tract and widespread in the environment. Until recently it had been presumed that the bacteria that colonise the endometrium are a random collection of many strains of *E. coli* from the gastrointestinal tract, urinary tract and environment. Interestingly, it now seems that specific strains of *E. coli* are adapted to the uterus and pathogenic in the endometrium, especially strains in phylogenetic groups A and B1 (Sheldon et al., 2010). *Escherichia coli* is rod-shaped, has a lipopolysaccharide (LPS) outer layer and some strains use flagella for motility (Lane et al., 2007). Thus, the TLRs likely to be important for recognition of *E. coli* include: TLR4, which binds LPS; TL5, which binds a component of flagellin, and TLR2, which binds lipoprotein elements of bacterial cell walls, such as lipoteichoic acid (Kawai and Akira, 2010).

It has previously been shown that LPS collects in the follicular fluid of cows with uterine disease, thus PAMPs are able to be in direct contact with GCs. Surprisingly, it was found that bovine GCs have functional responses to LPS, with treatment of GCs from emerged follicles with 1 or 10 μg/ml LPS significantly decreasing oestradiol production (Herath et al., 2007). In addition, GCs express mRNA for *TLR4* and associated adapter molecules, including *CD14* and *LY96* (Herath et al., 2007), supporting the hypothesis that GCs express functional *TLR4*. However, the expression of other TLRs and functional responses to other PAMPs have not yet been examined.

The Pam3CSK4 (PAM) synthetic triacylated lipopeptide binds TLR1/TLR2 dimers, and this work will use PAM as a TLR2 ligand due to the difficulty in obtaining ultrapure forms of other TLR2 ligands, such as bacterial peptidoglycan (PGN) and lipoteichoic acid (LTA). Flagellin (FLA) is a component of flagella found on gram negative and gram positive bacteria and is recognised by TLR5.

Ovulation has been likened to inflammation (Espey, 1980) and Toll-like receptors may play a role in events leading up to extrusion of the oocyte (Liu et al., 2008). This indicated that immune and endocrine pathways may interact, an idea backed up by the observations that the FSH receptor (FSHR) is necessary for innate immune responses in *Caenorhabditis elegans* (Powell et al., 2009) and that acute LH treatment of hen GCs increases expression of RNA for *TLR2* and *TLR4*. Hormones have also been linked with innate immune signalling: oestradiol has an inhibitory effect on TLR activation in murine uterine epithelial cells (Soboll et al., 2006) and suppresses LPS-induced NFκB activation and TNFα expression in human macrophages (Murphy et al., 2010) and progesterone decreases interleukin production by LPS-stimulated murine macrophages (Jones et al., 2008, Su et al., 2009). In this chapter, the crosstalk between the innate immune and endocrine pathways was further explored.

In addition to binding molecular patterns associated with pathogens, Toll-like receptors also bind patterns associated with damage. These may be important in the process of ovulation, which causes damage to the follicle, and also in the postpartum period, after extensive damage to the endometrium. Of particular interest is hyaluronic acid (HA), a major component of extracellular matrix, which is involved in wound repair, cell proliferation and migration. Hyaluronan is a major component of ovarian follicular fluid, is involved in oocyte maturation through interaction with CD44 and may aid capture of the oocyte by the oviduct (Salustri et al., 1999, Yokoo et al., 2010). In response to progesterone, the uterine lumen increases concentration of HA; this is maintained through early pregnancy (Ashworth et al., 1990). Thus, it is possible that HA could also travel to the ovary through the oviduct. During murine pregnancy, HA increases to 71% of total cervical glycoaminoglycan and during labour, activity of hyaluronidases increases, breaking down the high-molecular form of HA (Akgul et al., 2012). Thus, in the post-partum animal, the reproductive system contains a large amount of pro-inflammatory low-molecular weight HA, which may act as a DAMP in the ovary.

The hypothesis tested in this chapter was that GCs from emerged follicles respond to PAMPs and DAMPs, and that this response is influenced by endocrine factors. The aims of this chapter were to investigate the innate immune response of GCs from emerged follicles to 3 PAMPs (LPS, PAM and FLA) and 1 DAMP (HA), and to explore the interactions between ovarian physiology (the endocrine pathway) and pathology (the innate immune response). Firstly, expression and accumulation of pro-inflammatory cytokines and chemokines was measured after PAMP or DAMP exposure by qPCR and ELISA, respectively. Activation of TLR signalling components was identified using Western blot and the effect of blocking the TLR pathway was investigated using short interfering RNA (siRNA) and biochemical inhibitors. The effect of LPS and PAM on expression of endocrine receptors and production of oestradiol and progesterone was then identified, followed by the effect of endocrine factors on PAMP-stimulated pro-inflammatory cytokine production.

3.2 Methods

3.2.1 Granulosa cell culture

To explore the impact of PAMPs and DAMPs, GCs from emerged (4.0 to 7.5 mm) follicles from beef cows were isolated and plated in 12-, 24- or 96-well plates at a density of $1.5x10^6$ cells/ml in culture medium, as described in chapter 2. Granulosa cells from beef cattle represent a suitable model for investigating the effects of infection and inflammation on dairy GCs (chapter 2). Furthermore, GCs from follicles 4.0 to 7.5 mm in diameter represented GCs from emerged follicles, with expression of *FSHR*, low expression of *CYP19A1* and no detectable expression of *LHCGR* (chapter 2). The GC cultures were also free from contaminating immune cells, thus validating follicle aspiration as a suitable method for GC isolation. Bovine PBMCs were isolated from whole blood as described in chapter 2 and used as a positive control in some experiments.

After 48 h, the GCs were washed in PBS, then treated with control medium or medium containing ultrapure lipopolysaccharide from *Escherichia coli* O111:B4 (LPS; Invivogen), Pam3CSK4 (PAM; Invivogen), flagellin from *Salmonella typhimurium* (FLA; Invivogen) or ultralow (ULMW) or high molecular weight (HMW) hyaluronic acid (both R&D). Cellular responses of GCs were tested using 10-fold increasing concentrations from 0.001 to 10 μg/ml LPS or PAM and 0.0001 to 1μg/ml FLA. The response of GCs to HA was tested in the presence or absence of 1 μg/ml LPS, using 10-fold increasing concentrations from 0.1 to 1000 μg/ml ULMW or HMW HA. In subsequent experiments using PAMPs, GCs were challenged with 1 μg/ml LPS, 1 μg/ml PAM or 0.1 μg/ml FLA for 3 or 24 h for analysis by qPCR or ELISA, respectively; or for up to 5 to 25 min for analysis by Western blot.

To investigate the interaction between endocrine and immune pathways, GCs were treated with 100 ng/ml FSH (Henderson et al., 1982, McLaughlin et al., 2010), 4 ng/ml LH (Henderson et al., 1982, Fortune and Hansel, 1985), 500 ng/ml oestradiol (E2; Sigma) (Ginther et al., 2001, Beg et al., 2002), 250 ng/ml progesterone (P4; Sigma) (Martin et al., 1991, Ginther et al., 2001) or 10 ng/ml

epidermal-like growth factor (EGF; Calbiochem) (Langhout et al., 1991, Park et al., 1997) in combination with 1 μg/ml LPS or PAM.

At the end of each experiment, supernatants were collected and stored at -20°C prior to protein analysis by ELISA; cells were washed with PBS and an MTT test carried out to estimate cell viability, as described in chapter 2. For experiments where the method of analysis was qPCR or Western blot, cells were washed twice with PBS and then lysed using 350 μl buffer RLT or 80 μl Phosphosafe, respectively.

3.2.2 Chemical inhibitors

To explore the role of intracellular signalling pathways in the response of GCs to PAMPs, GCs were seeded in a 96-well plate (TPP), equilibrated for 48 h and washed with PBS. The GCs were then treated for 30 min with 50 μl of medium containing a range of concentrations of inhibitors targeting NFκB (0.0008-80 μM), PKC (0.002-200 μM), JNK (0.01-1000 μM), p38 MAPK (0.002-200 μM) or MEK (0.002-20 μM; all Calbiochem) or DMSO (1:500). This 30 minute treatment was followed by addition of 50 μl control medium, or 50 μl medium containing 2 μg/ml LPS or 2 μg/ml PAM. In a further experiment, to limit the impact of inhibitors on cell health, inhibitors were also used at their lowest concentration in combination with each other using pairs of 25 μl inhibitors targeting NFκB (0.0016 μM), PKC (0.004 μM), JNK (0.02 μM), p38 MAPK (0.004 μM) or MEK (0.004 μM) for 30 min, followed by 50 μl of medium containing LPS or PAM treatment as above.

3.2.3 Short-interfering RNA

To explore the role of TLRs, GCs were transfected with short-interfering RNA targeting *TLR2* or *TLR4* (as described in general methods). After 24 h, cells were washed with PBS and lysed using buffer RLT for analysis of TLR expression by qPCR, or treated with control medium or medium containing 1 μg/ml LPS or PAM for 24 h. Supernatants were collected after 24 h and stored at -20°C for analysis by ELISA; cell viability was assessed by MTT assay, as described in chapter 2.

3.2.4 ELISA

Accumulation of IL-6, IL-8, IL-1β and TNFα was measured in the supernatant of GCs treated with LPS, PAM or FLA, as described in general methods (chapter 2), using kits from R&D Systems for IL-8 and TNFα and kits from Thermo Fisher Scientific for IL-6 and IL-1β. Two technical replicates were used for each sample in each ELISA, from at least 3 experiments.

3.2.5 RNA extraction, cDNA synthesis and PCR

Granulosa cells were treated with control medium or medium containing 1 μg/ml LPS, 1 μg/ml PAM or 0.1 μg/ml FLA for 3 h. Cells were washed twice in PBS, lysed using 600 μl buffer RLT and scraped using a cell scraper. Total RNA was extracted using the RNeasy Mini kit, as described in general methods. Total RNA was quantified using a Nanodrop spectrophotometer and 1 μg total RNA used to synthesise cDNA, using the QuantiTect kit, as described in general methods. Expression of mRNA encoding genes considered as markers of an innate immune response (*IL6, IL1B, TNF, IL10, IL8, CCL5*) and endocrine receptors (*FSHR, EGFR*) was measured by qPCR, using 3 technical replicates for each sample from 3-4 experiments.

3.2.6 Western blot

In order to investigate the activation of ERK1/2 and p38 MAPK, GCs were plated in a 12-well plate, equilibrated for 24 h, washed twice with PBS and cultured in Optimem (low serum medium) for 24 h. They were then treated for 0, 5, 10, 15, 20 or 25 min with control Optimem medium, or Optimem containing 1 μg/ml LPS or 1 μg/ml PAM. The medium was discarded before the GCs were washed with PBS and 80 μl Phosphosafe Extraction Reagent (Novagen) was used to lyse each well of cells. Protein was extracted and quantified using the DC assay (chapter 2), before blotting for diphosphorylated ERK1/2 (1:1000; Sigma 8159) or phosphorylated p38 MAPK (1:1000; Acris Antibodies, San Diego, USA; APO5898PU-N). After imaging each blot using the ChemiDoc XRS System, gels were stripped for 7 min in Restore Western Blot Stripping Buffer and then re-stained for β-actin (1:1000; Abcam, Cambridge, UK; ab8226), as described in general methods.

Images of each blot were analysed using the ChemiDoc XRS System to measure peak density of each band, after adjusting for background, as described in chapter 2. Blots from 3 experiments were quantified, with 1 image shown as a representative in the results section.

3.2.7 Statistical analyses

Data are presented as mean + SEM, with statistical analyses performed using SPSS (version 16, SPSS Inc) and significance ascribed when $P < 0.05$. Analysis of variance was used to examine normally distributed data, following square root or log transformation where indicated in Results, as necessary. Dunnett's pairwise multiple comparison T-test was used to compare treatments with control. Data that is not significantly different by ANOVA are denoted with the same letter. Where data were not normally distributed as indicated in Results, comparisons between treatment and control were made using Mann Whitney U Test.

3.3 Results

3.3.1 Granulosa cells expressed mRNA for TLRs

The GCs from emerged follicles were negative for MHCII by FACS and did not express *MHCII* or *CD45* mRNA (chapter 2), indicating they were not contaminated by immune cells. Using these cells, the expression of TLRs was analysed by qPCR and the efficiencies of all primers were 100% ± 10%. All 10 TLRs were expressed by GCs, with higher expression of *TLR3* compared to all other TLRs ($P < 0.001$) and lower expression of *TLR8* compared to the expression of mRNA encoding TLRs 1-7 (Fig. 3.1A; $P < 0.05$). Mononuclear cells were used as a positive control; the expression of each TLR was not significantly different to any other TLR (Fig 3.1B; $P > 0.23$).

In order to investigate whether treatment of GCs with one PAMP primed the cells to respond to another PAMP, TLR expression after the cells were treated with LPS or PAM was examined. Treatment of GCs for 3 h with LPS increased expression of mRNA encoding *TLR2, TLR3* and *TLR5* and decreased expression of *TLR7.* Treatment of GCs for 3 h with PAM also increased expression of mRNA encoding *TLR2* and decreased expression of *TLR4, TLR6, TLR7* and *TLR10* (Fig. 3.2A). To examine whether this change in mRNA levels corresponded to a functional change at the protein level, GCs were treated for 3 h with control medium or medium containing LPS or PAM. They were then washed and cultured for a further 24 h with control medium or medium containing LPS or PAM. Pre-treating with PAM for 3 h increased accumulation of IL-6 in cells cultured in control medium for the subsequent 24 h, but there were no other significant effects of pre-treatment (Fig. 3.2B).

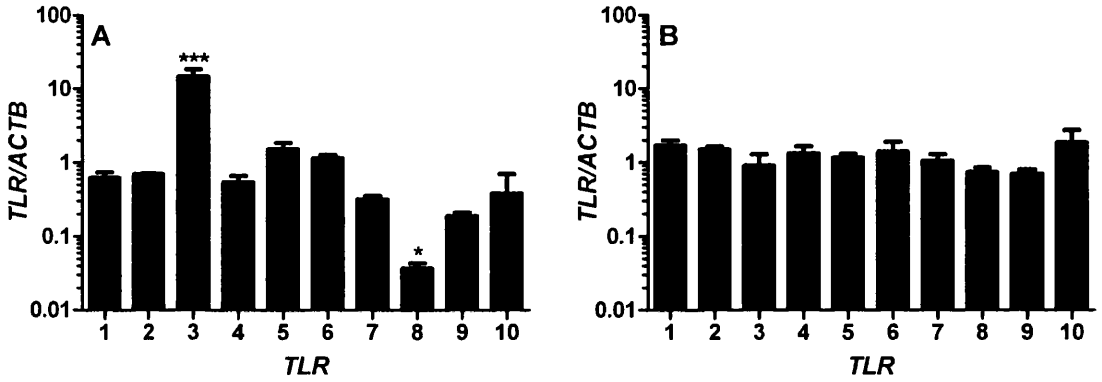


Figure 3.1 Granulosa cells expressed *TLRs 1-10*. Granulosa cells were isolated from emerged follicles (A) and bovine peripheral blood mononuclear cells (B). RNA was extracted and expression of TLRs measured by qPCR. Data represented as mean + SEM, normalised to *ACTB* and represent 3 independent experiments. Primer efficiencies were 100% ± 10% for each primer pair. Expression varies from all other TLRs by ANOVA and a post-hoc Fisher's least significant difference test (using $\log_{10}$ normalised data), * $P < 0.05$ *** $P < 0.001$

A

TLR	LPS		PAM	
	Fold	P	Fold	P
TLR1	0.81±0.08		0.77±0.08	
TLR2	1.93±0.31	*	2.92±0.47	**
TLR3	2.43±0.80	*	0.86±0.33	
TLR4	1.25±0.19		0.75±0.11	*
TLR5	2.21±0.58	*	0.72±0.31	
TLR6	0.97±0.06		0.71±0.12	**
TLR7	0.41±0.08	***	0.30±0.04	***
TLR8	0.55±0.12		0.51±0.16	
TLR9	1.14±0.35		0.71±0.11	
TLR10	0.83±0.10		0.56±0.09	**

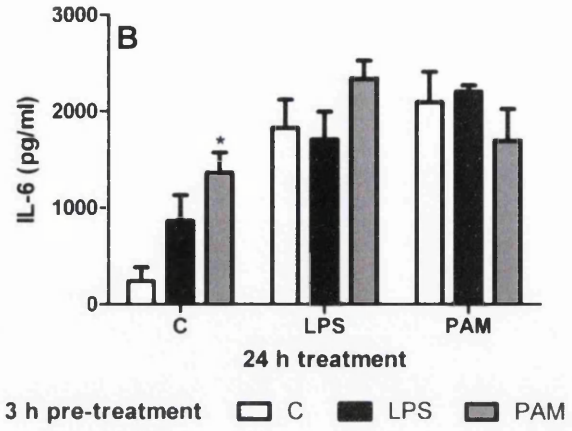


Figure 3.2 Pre-treatment of GCs with LPS or PAM impacted TLR expression. (A) Granulosa cells were treated for 3 h with control medium or medium containing 1 μg/ml LPS or PAM. The expression of mRNA encoding TLRs 1-10 was measured by qPCR and is expressed as fold change over control, relative to *ACTB*. Data presented as mean + SEM and represent 3 independent experiments. Values differ from control by Student's T-test using $\log_{10}$-normalised data; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. In a subsequent experiment, GCs were pre-treated for 3 h (as above), then further treated for 24 h with control medium ('C') or medium containing 1 μg/ml LPS or PAM. Supernatants were collected and the accumulation of IL-6 measured by ELISA (B). Data presented as mean + SEM and represent 4 independent experiments. Values differ from cells pre-treated with control medium within subsequent treatment group by ANOVA; $P < 0.05$.

3.3.2 Granulosa cells responded to PAMPs

When GCs were treated for 24 h with control medium or medium containing 10-fold increasing concentrations of LPS, PAM or FLA, supernatants accumulated IL-6, IL-1β, IL-8 and TNFα. Treatment with LPS significantly increased accumulation of IL-6 and IL-8 at concentrations ≥ 0.01 μg/ml LPS (Fig. 3.3A, D) and increased accumulation of IL-1β even from the lowest concentration tested (Fig. 3.3B). However there was only a significant increase in the accumulation of TNFα at 10 μg/ml LPS treatment (Fig. 3.3C). The GCs exhibited a similar response to PAM, with increased accumulation of IL-6, IL-1β and IL-8 at concentrations ≥ 0.01 μg/ml PAM (Fig. 3.4A, B, D) and increased accumulation of TNFα at 10 μg/ml PAM (Fig. 3.4C). Interestingly, although the accumulation of IL-6, TNFα and IL-8 was a similar concentration whether cells were treated with LPS or PAM, treatment with PAM resulted in a lower concentration of IL-1β than treatment with LPS. Treatment with FLA did not increase accumulation of IL-1β, IL-8 or TNFα (all below limits of detection). FLA treatment did significantly increase IL-6 at ≥ 0.1 μg/ml FLA; however, even at 1 μg/ml FLA, accumulation of IL-6 was only 46 ± 10 pg/ml, compared to 1407 ± 267 pg/ml after LPS stimulation and the lowest standard on the kit of 78 pg/ml. For future experiments, 1 μg/ml LPS or PAM and 0.1 μg/ml FLA were used as the standard working concentrations. For FLA this was the highest feasible concentration to use (1 μg/ml would have been too costly) and the concentration of 0.1 μg/ml stimulated a significant increase in IL-6. For LPS and PAM, 1 μg/ml stimulated the greatest accumulation of IL-6, and 1 μg/ml LPS is similar to concentrations of LPS previously measured in the follicular fluid of cows with severe uterine disease (Herath et al., 2007).

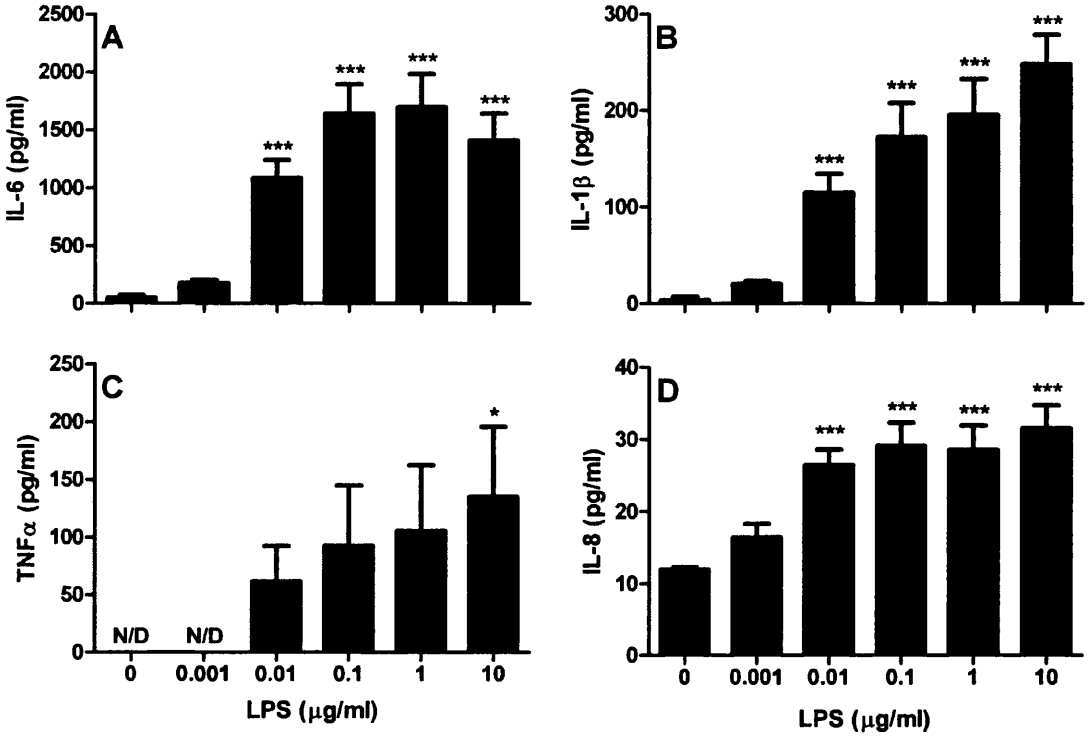


Figure 3.3 Granulosa cells responded to LPS in a concentration-dependent manner. Granulosa cells from emerged follicles were treated for 24 h with control medium ('0') or medium containing 0.001, 0.01, 0.1, 1 or 10 μg/ml ultrapure LPS from *E. coli* 0111:B4. Supernatants were collected and the accumulation of IL-6 (A), IL-1β (B), TNFα (C) and IL-8 (D) measured by ELISA. Data presented as mean + SEM and represent at least 3 independent experiments. Values differ from control by ANOVA and Dunnett's pairwise multiple comparison t test (square root normalised data for A and B; $\log_{10}$ normalised data for (C) or Mann Whitney U test (D) * $P < 0.05$, *** $P < 0.001$. N/D = below limits of detection.

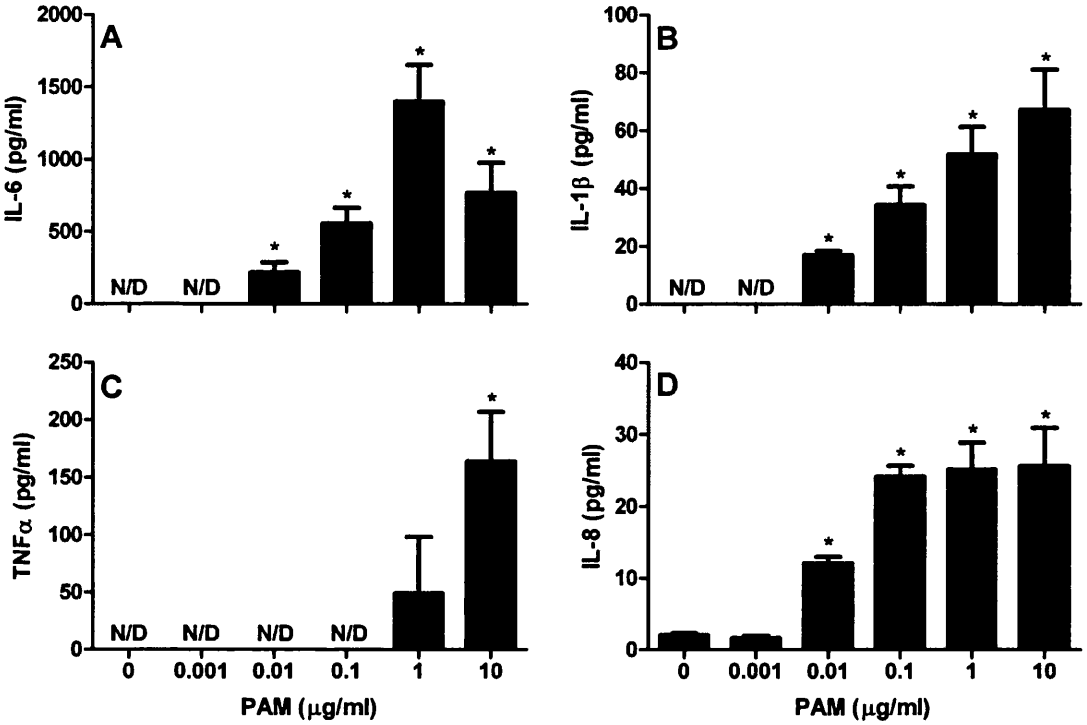


Figure 3.4 Granulosa cells responded to PAM in a concentration-dependent manner. Granulosa cells from emerged follicles were treated for 24 h with control medium ('0') or medium containing 0.001, 0.01, 0.1, 1 or 10 μg/ml Pam3CSK4 (PAM). Supernatants were collected and the accumulation of IL-6 (A), IL-1β (B), TNFα (C) and IL-8 (D) measured by ELISA. Data presented as mean + SEM and represent 3-5 independent experiments. Values differ from control by Mann Whitney U test * $P < 0.05$. N/D = below limits of detection.

3.3.3 Granulosa cells did not mount an inflammatory response to HA

When GCs were treated for 24 h with control medium or medium containing 10-fold increasing concentrations of ULMW or HMW HA in the presence or absence of LPS, there was no effect of HA on the accumulation of IL-6. Indeed the concentrations of IL-6 were below the limits of detection for control and cells treated with ULMW or HMW HA (Fig. 3.5A, B). However, as expected, GCs treated with LPS accumulated IL-6 at all concentrations of HA ($P < 0.001$). When 1 mg/ml HMW HA was used in combination with LPS, there was a significant decrease in accumulation of IL-6 ($P = 0.05$; Fig. 3.5B).

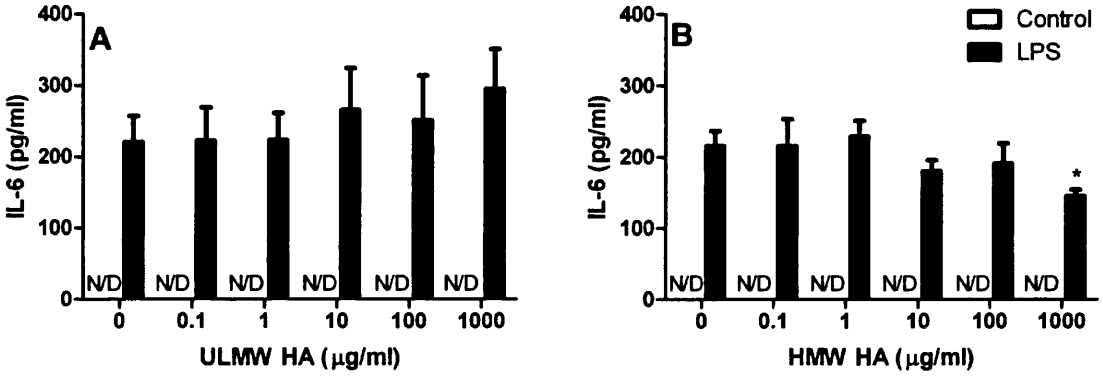


Figure 3.5 Granulosa cells did not mount an inflammatory response to HA. Granulosa cells from emerged follicles were treated for 24 h with control medium ('0') or medium containing 10-fold increasing concentrations of ULMW (A) or HMW (B) HA in the absence (open bars) or presence of 1 μg/ml LPS (black bars). Supernatants were collected and the accumulation of IL-6 measured by ELISA. Data represent mean + SEM of at least 3 independent experiments. Values differ from 0 μg/ml HA in combination with 1 μg/ml LPS by Mann Whitney, * $P \leq 0.05$. N/D = below limits of detection.

3.3.4 Exposure of GCs to PAMPs increased expression of genes associated with inflammatory or innate immune pathways

When GCs were treated for 3 h with control medium or medium containing LPS (Fig. 3.6A-F), PAM (Fig. 3.7A-F) or FLA (Fig. 3.8A-F), there was increased expression of *IL6, IL1B, IL10, TNF, IL8* and *CCL5*. All 6 of these genes associated with inflammatory or innate immune pathways had similar fold-increases for each replicate (Fig. 3.6 - 3.9 where each line represents one experiment, with the same colour points representing the same experiment across figures 3.6-3.9). In general, experimental replicates with high expression of one cytokine also had high expression of all other cytokines measured. Treatment with LPS increased expression of *TNF* ($P < 0.05$) and *CCL5* ($P < 0.001$) and tended towards increased expression of *IL10* ($P = 0.051$) to a greater extent compared to treatment with FLA, and increased expression of *CCL5* ($P < 0.001$) to a greater extent than treatment with PAM (Fig. 3.9). Treatment with PAM increased expression of *IL1B* ($P < 0.05$), *IL10* ($P < 0.01$), *TNF* ($P < 0.01$) and tended towards increasing expression of *IL6* ($P = 0.065$) to a greater extent than treatment with FLA and increased expression of *IL1B* and *IL8* ($P < 0.05$) to a greater extent than LPS treatment. Treatment with FLA did not stimulate a greater increase in any cytokine measured than treatment with LPS or PAM. Therefore, as FLA did not yield protein responses, LPS and PAM treatment only were used for further experiments. There was no significant effect of LPS ($P=0.626$) or PAM ($P=0.582$) on *ACTB* expression.

3.3.5 Exposure of GCs to PAM, but not LPS, increased expression of genes associated with endocrine function

There was no significant effect of LPS (Fig. 3.10A, E) or PAM (Fig. 3.10B, F) on expression of *FSHR*. Treatment with PAM, but not LPS, increased expression of *EGFR* (Fig. 3.10D, F).

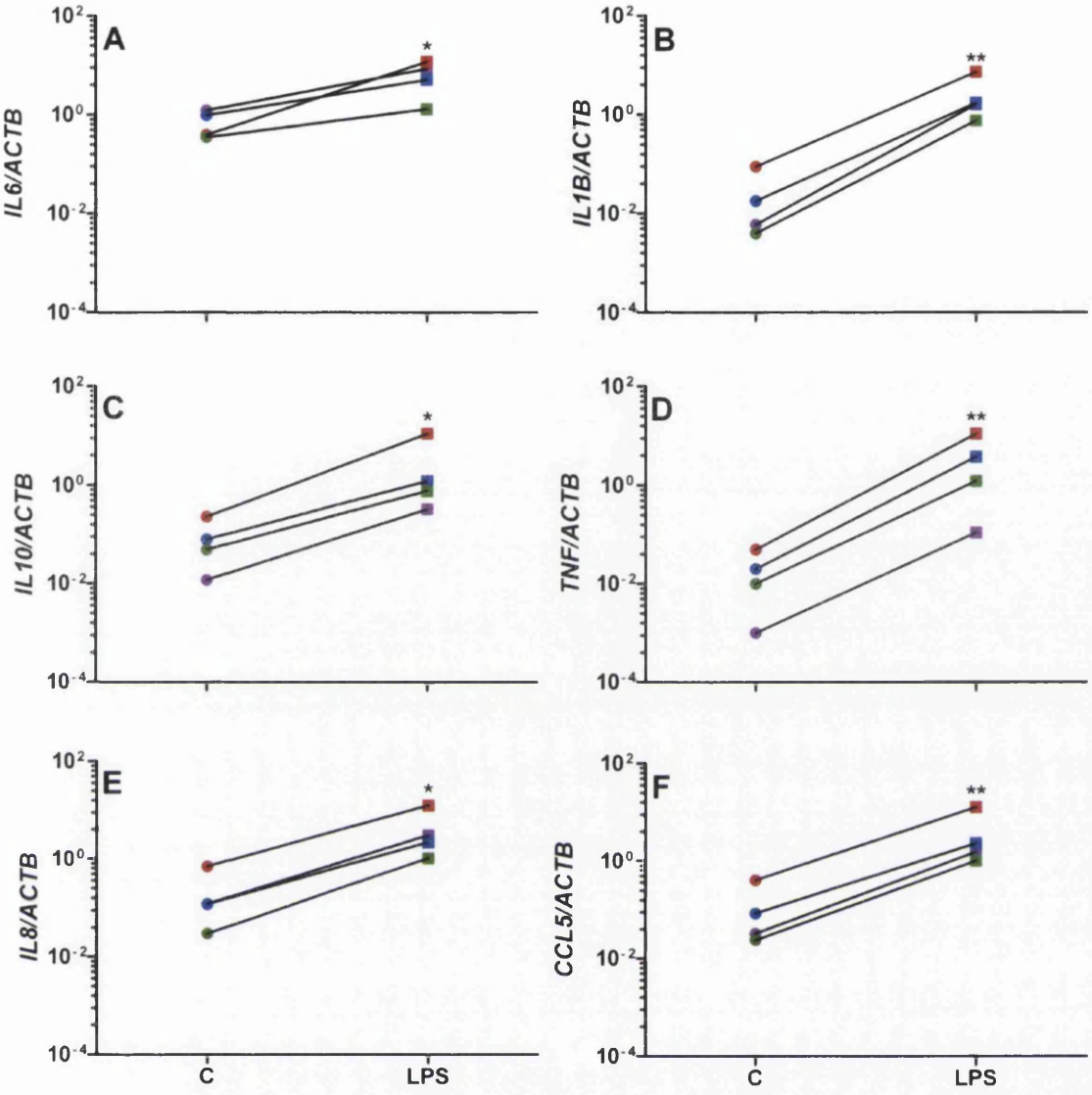


Figure 3.6 Exposure of GCs to LPS increased expression of genes associated with inflammation and innate immunity. Granulosa cells from emerged follicles were treated for 3 h with control medium ('C') or medium containing 1 μg/ml ultrapure lipopolysaccharide from *E. coli* 0111:B4 (LPS). RNA was extracted, cDNA synthesised and the expression of *IL6* (A), *IL1B* (B), *IL10* (C), *TNF* (D), *IL8* (E) and *CCL5* (F) estimated by qPCR. Each independent experiment is presented as one line, with coloured dots representing the same experiment throughout. Mean values for LPS treatment differ from control by T-test using log-normalised data, * $P < 0.05$, ** $P < 0.01$

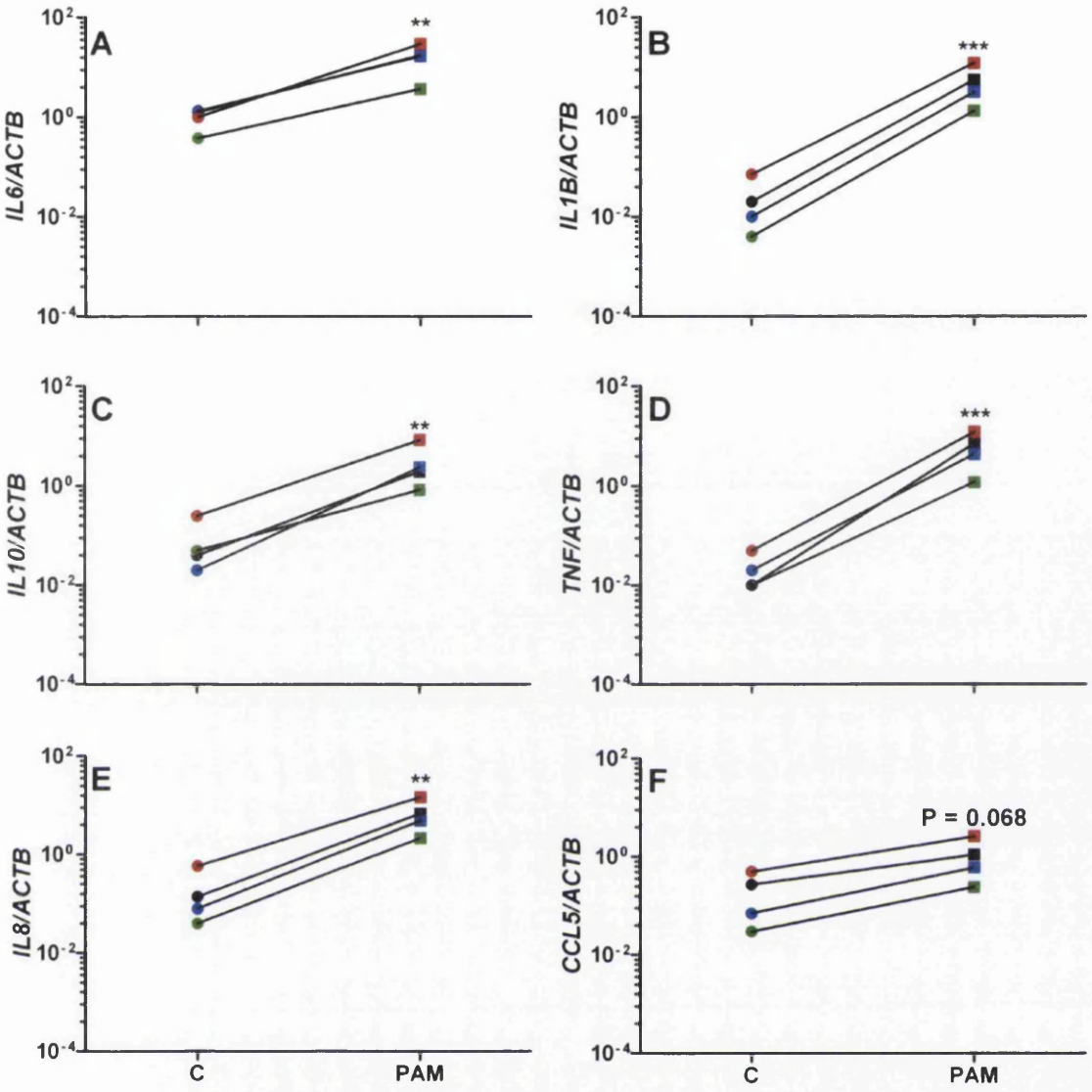


Figure 3.7 Exposure of GCs to PAM increased expression of genes associated with inflammation and innate immunity. Granulosa cells from emerged follicles were treated for 3 h with control medium ('C') or medium containing 1 μg/ml Pam3CSK4 (PAM). RNA was extracted, cDNA synthesised and the expression of *IL6* (A), *IL1B* (B), *IL10* (C), *TNF* (D), *IL8* (E) and *CCL5* (F) estimated by qPCR. Each independent experiment is presented as one line, with coloured dots representing the same experiment throughout. Mean values for PAM treatment differ from control by T-test using log-normalised data, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

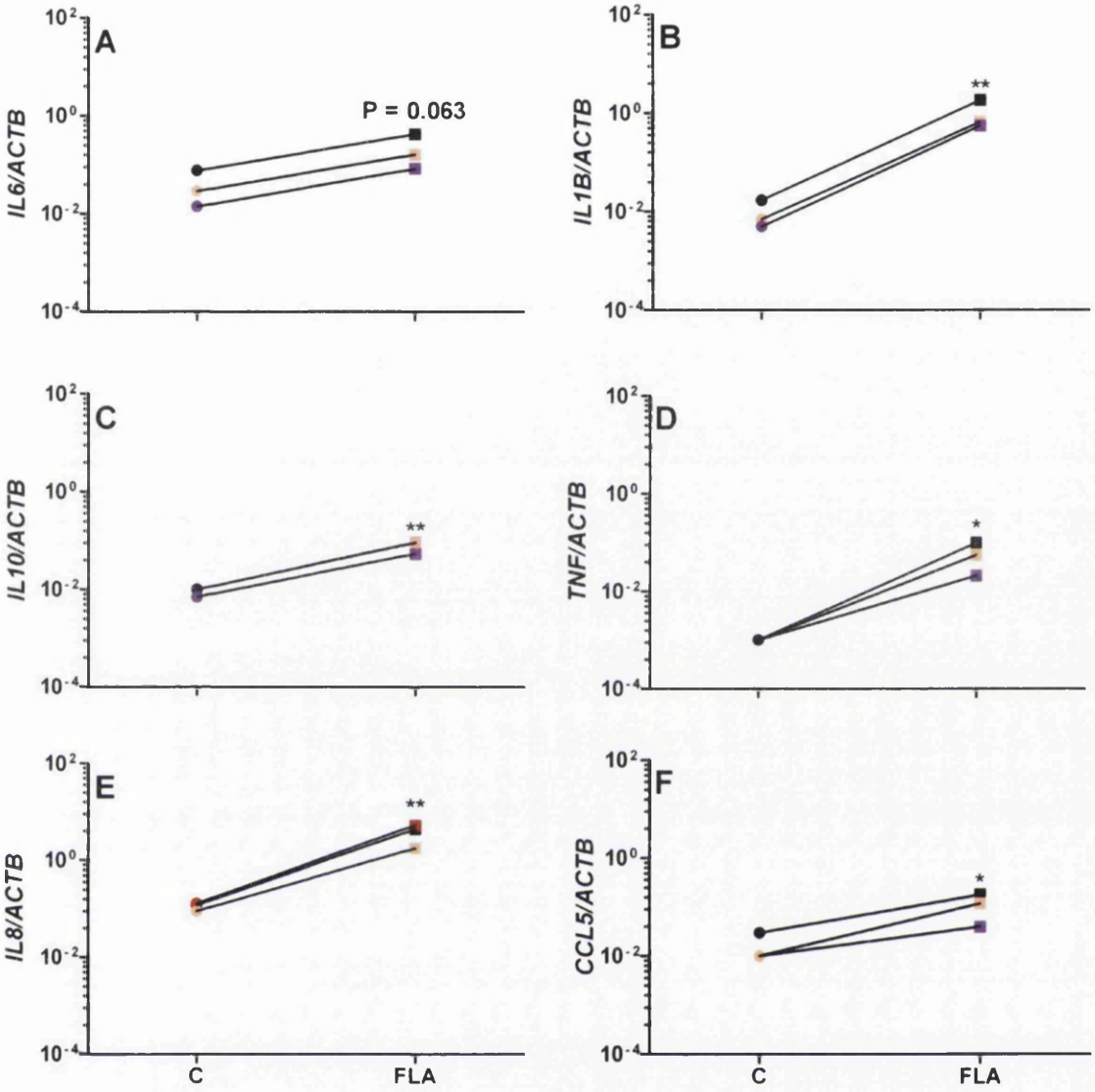


Figure 3.8 Exposure of GCs to FLA increased expression of genes associated with inflammation and innate immunity. Granulosa cells from emerged follicles were treated for 3 h with control medium ('C') or medium containing 0.1 μg/ml ultrapure flagellin (FLA). RNA was extracted, cDNA synthesised and the expression of *IL6* (A), *IL1B* (B), *IL10* (C), *TNF* (D), *IL8* (E) and *CCL5* (F) estimated by qPCR. Each independent experiment is presented as one line, with coloured dots representing the same experiment throughout. Mean values for FLA treatment differ from control by T-test using log-normalised data, * $P < 0.05$, ** $P < 0.01$

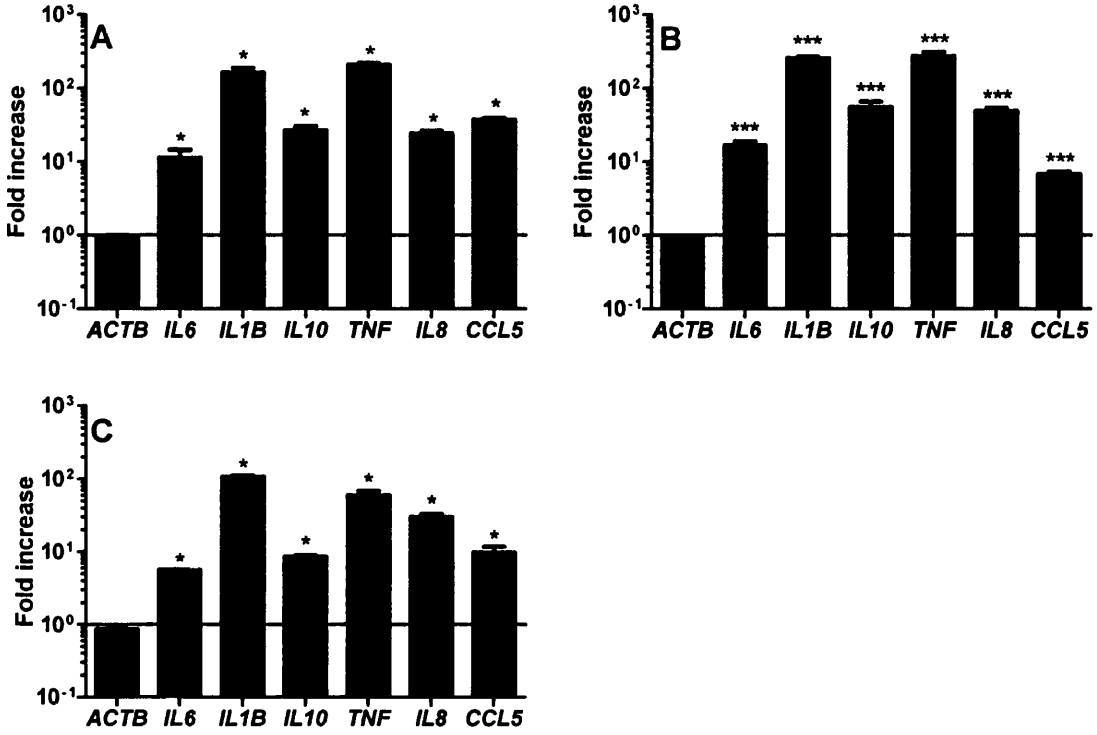


Figure 3.9 Exposure of GCs to LPS, PAM or FLA increased expression of genes associated with inflammation and innate immunity. Granulosa cells from emerged follicles were treated for 3 h with control medium or medium containing 1 μg/ml ultrapure lipopolysaccharide from *E. coli* 0111:B4 (A), 1 μg/ml Pam3CSK4 (B) or 0.1 μg/ml flagellin (C). RNA was extracted, cDNA synthesised and the expression of *ACTB, IL6, IL1B, IL10, TNF, IL8* and *CCL5* estimated by qPCR. Data are presented as mean fold increase in each gene compared to control. Data represented as mean + SEM of 3-4 independent experiments. Values differ from control by Mann Whitney (A, C) or ANOVA and Dunnett's post hoc multiple comparison T-test (B), * $P < 0.05$, *** $P < 0.001$

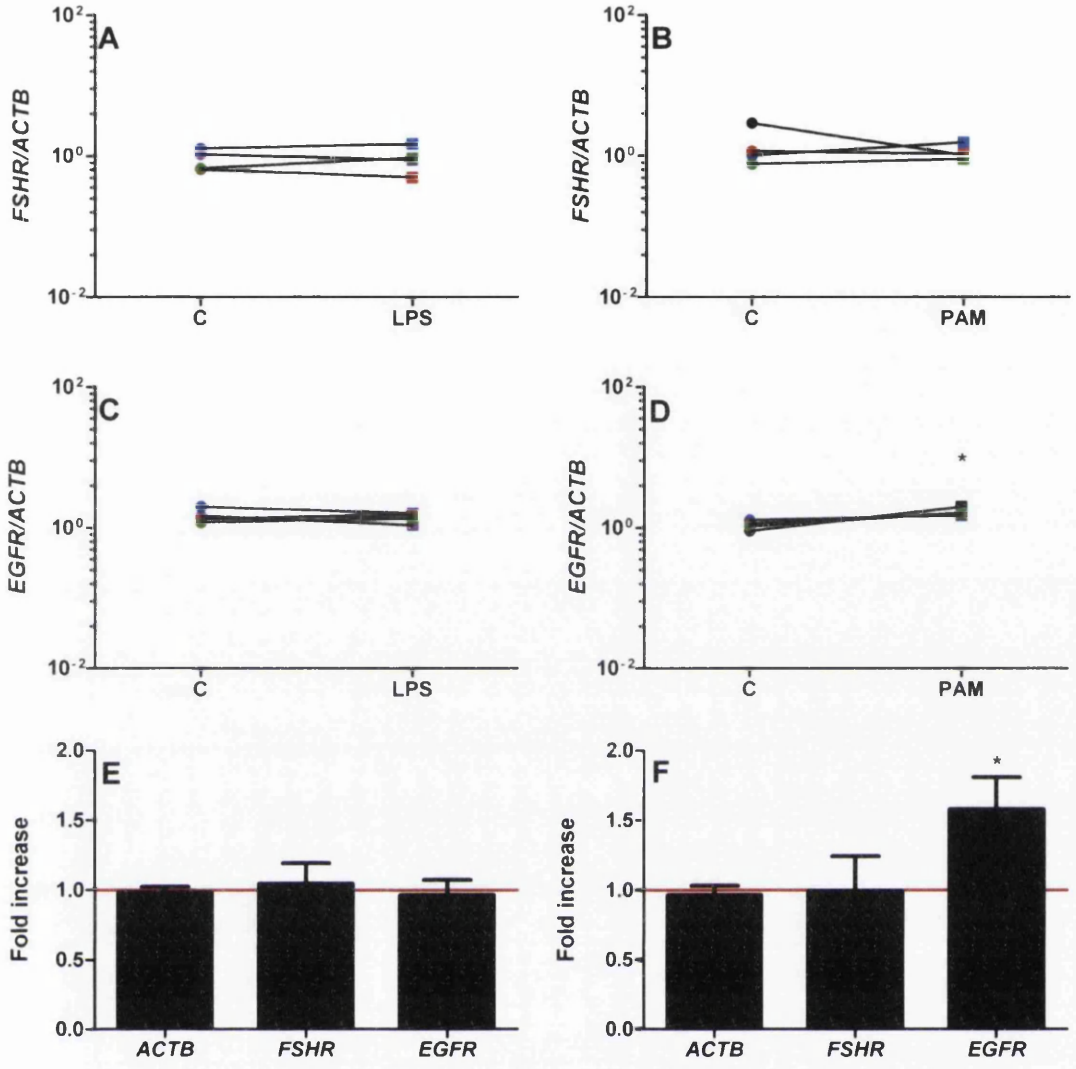


Figure 3.10 Exposure of GCs to PAM, but not LPS, increased expression of genes encoding endocrine receptors. Granulosa cells from emerged follicles were treated for 3 h with control medium ('C') or medium containing 1 μg/ml lipopolysaccharide (A, C, E; LPS) or Pam3CSK4 (B, D, F; PAM). RNA was extracted, cDNA synthesised and the expression of *FSHR* (A, B) and *EGFR* (C, D) estimated by qPCR. Each independent experiment is presented as one line, with each colour dot representing the same experiment throughout. (E, F) Data are presented as mean fold increase in each gene compared to control. Data presented as mean + SEM and represent 4 independent experiments. Values differ from control by Mann Whitney U test, * $P < 0.05$.

3.3.6 Treatment of GCs with PAMPs activated intracellular signalling pathways

When GCs were treated with control medium or medium containing PAMPs, 15 min LPS treatment increased phosphorylation of p38 MAPK and ERK1/2 (Fig. 3.11A-C). After 5 min PAM treatment, there was a trend towards increased phosphorylation of p38 MAPK ($P < 0.09$, Fig. 3.12A, B) and a significant increase in phosphorylation of ERK1/2 (Fig. 3.12C), which was maintained for 15 min.

3.3.7 The response of GCs to PAMPs was attenuated using short-interfering RNA targeting TLRs

When GCs were treated with lipofectamine vehicle, scrambled siRNA or siRNA targeting *TLR2* or *TLR4* for 24 h, there was no effect of siRNA on cell viability as evaluated by MTT test ($P > 0.5$; Fig. 3.13A). Treatment with each targeted siRNA significantly decreased expression of *TLR2* or *TLR4* mRNA ($P = 0.05$; Fig. 3.13B) and treatment with scrambled siRNA did not significantly affect expression of either target gene ($P > 0.2$). Treatment with PAM significantly increased the accumulation of IL-6, but this was attenuated when cells were pre-treated with siRNA targeting *TLR2* (Fig. 3.13C). Treatment with LPS significantly increased the accumulation of IL-6, but this was also attenuated when cells were pre-treated with siRNA targeting *TLR4* (Fig. 3.13D). There was no effect of lipofectamine vehicle, scrambled siRNA or targeted siRNA on accumulation of IL-6 by GCs treated with control medium.

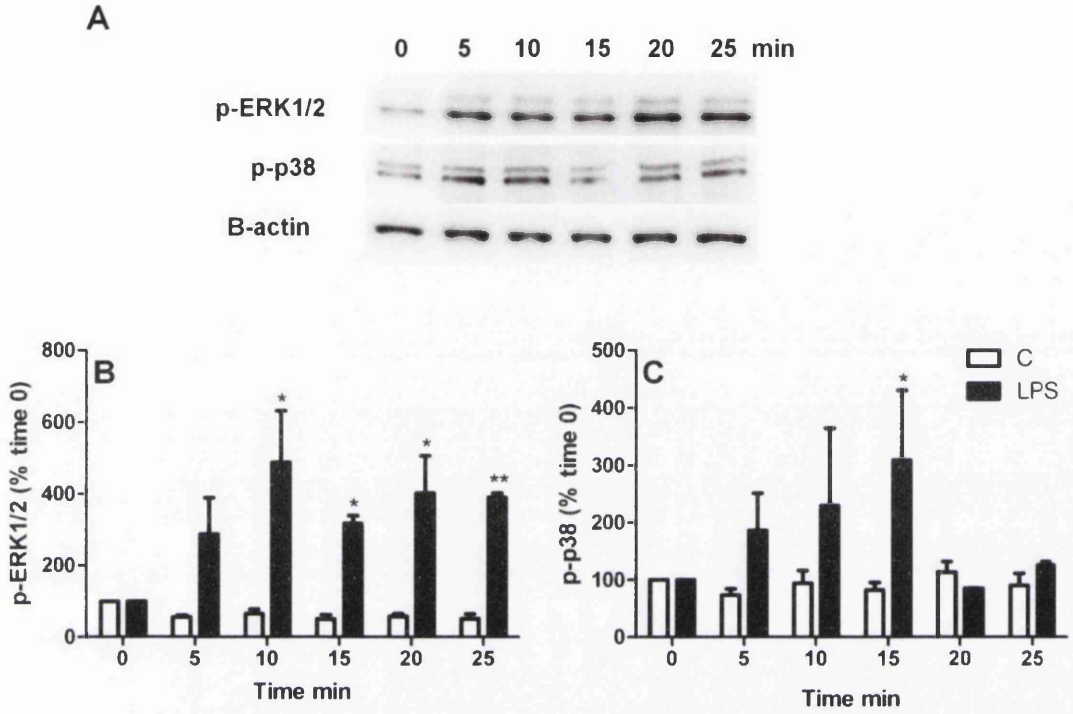


Figure 3.11 LPS induced phosphorylation of ERK1/2 and p38 MAPK in GCs. Granulosa cells from emerged follicles were treated for 5, 10, 15, 20 or 25 mins with control medium (open bars) or medium containing 1 μg/ml LPS (black bars) and phosphorylation of ERK1/2 and p38 MAPK analysed by Western blot. (A) A representative blot showing LPS treatment with bands corresponding to diphosphorylated ERK1/2, phosphorylated p38 MAPK (Thr180/182) and β-actin. Band densities were quantitated relative to β-actin and phosphorylation of ERK1/2 (B) and p38 MAPK (C) expressed as a percentage of time 0. Data presented as mean + SEM and represent 3 independent experiments. Values differ from control at each time point by t-test (log_{10} normalised data), * $P < 0.05$, ** $P < 0.01$

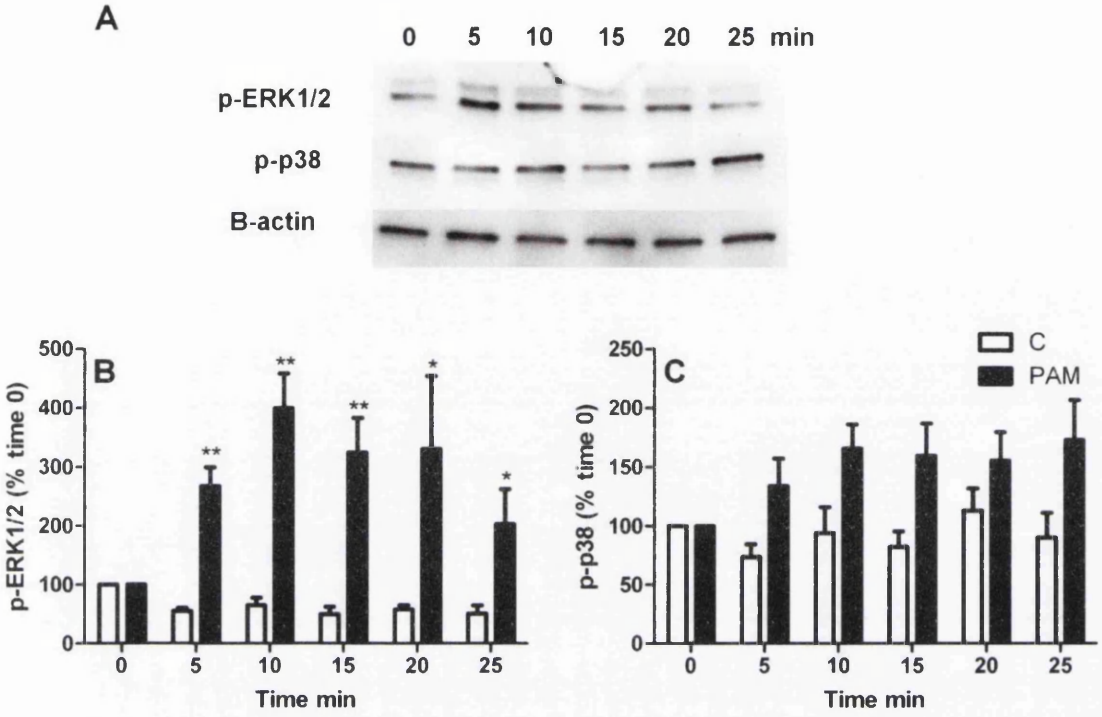


Figure 3.12 PAM induced phosphorylation of ERK1/2 in GCs. Granulosa cells from emerged follicles were treated for 5, 10, 15, 20 or 25 mins with control medium (open bars) or medium containing 1 μg/ml PAM (black bars) and phosphorylation of ERK1/2 and p38 MAPK analysed by Western blot. (A) A representative blot showing PAM treatment with bands corresponding to diphosphorylated ERK1/2, phosphorylated p38 MAPK (Thr180/182) and β-actin. Band densities were quantitated relative to β-actin and phosphorylation of ERK1/2 (B) and p38 MAPK (C) expressed as a percentage of time 0. Data presented as mean + SEM and represent 3 independent experiments. Values differ from control at each time point by t-test (log_{10} normalised data), * $P < 0.05$, ** $P < 0.01$

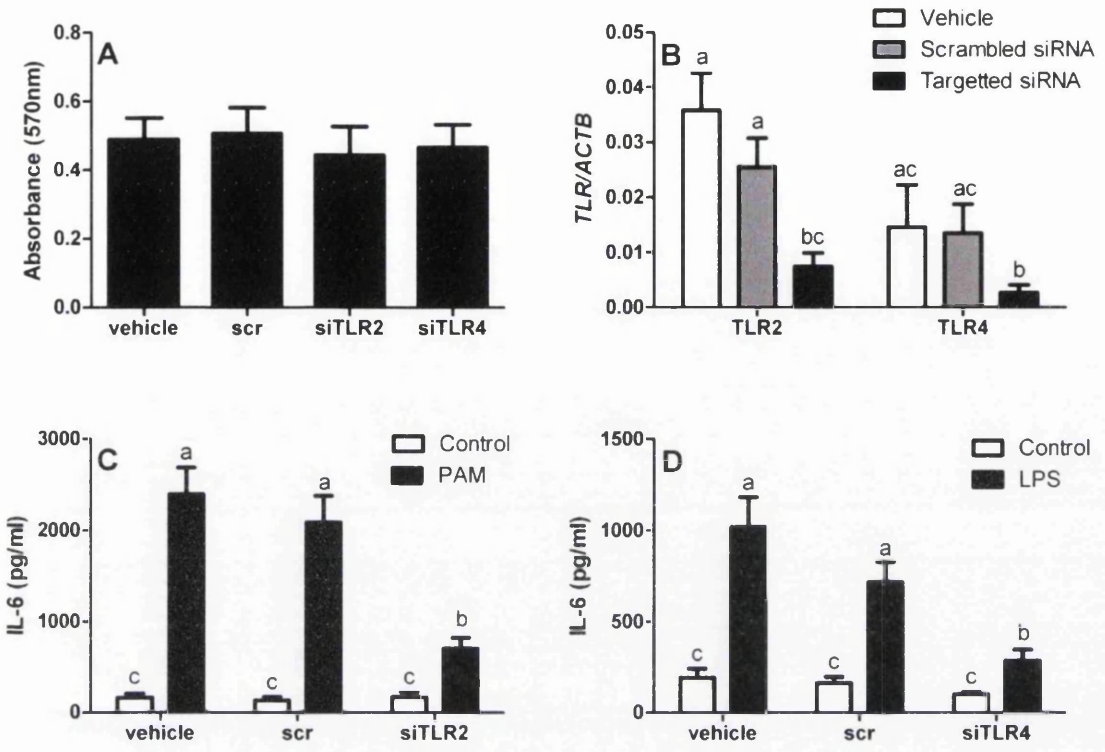


Figure 3.13 The IL-6 response of GCs to LPS or PAM was attenuated using siRNA targeting TLR4 or TLR2, respectively. Granulosa cells from emerged follicles were treated for 24 h with lipofectamine ('vehicle'), scrambled siRNA ('scr'), siRNA targeting *TLR2* ('siTLR2') or targeting *TLR4* ('siTLR4'). After washing, the cells were treated for 24 h with control medium (white bars) or medium containing 1 μg/ml LPS or 1 μg/ml PAM (black bars). (A) Cell viability was examined by MTT test. (B) Expression of *TLR2* and *TLR4* mRNA was examined by qPCR and normalised to the *ACTB* reference gene. Values differ by Mann Whitney U test. Accumulation of IL-6 was measured in the cell supernatants after treatment with PAM (C) or LPS (D). Values denoted by the same letter do not differ by Mann Whitney U test ($P > 0.05$). Data presented as mean + SEM and represent 3-5 independent experiments.

3.3.8 EGF increased accumulation of IL-6 by GCs

To examine the effect of the endocrine environment, GCs were treated for 24 h with control medium (Fig. 3.14) or medium containing 1 μg/ml LPS (Fig. 3.15) or PAM (Fig. 3.16) in combination with 100 ng/ml FSH, 4 ng/ml LH, 500 ng/ml E2, 250 ng/ml P4 or 10 ng/ml EGF. Treatment with EGF significantly increased accumulation of IL-6 by GCs treated with control medium or PAM (Fig. 3.14A, 3.16A; $P < 0.05$), and there was a trend towards increased accumulation of IL-6 by GCs treated with LPS (Fig. 3.15A; $P < 0.15$). Oestradiol treatment significantly decreased IL-6 accumulation by GCs treated with control medium ($P < 0.01$), but had no effect on IL-6 accumulation stimulated by LPS or PAM. There was no significant effect of FSH, LH or P4 had on IL-6 accumulation by GCs. There was no effect of endocrine treatment on accumulation of IL-1β (Fig. 3.14-16B; $P > 0.9$) or IL-8 (Fig. 3.14-16C; $P > 0.9$), or on cell viability measured by MTT test (Fig. 3.14D; treatments in control medium $P > 0.9$; Fig 3.15D; treatments in medium containing LPS $P > 0.7$; Fig 3.16D; treatments in medium containing PAM $P > 0.3$).

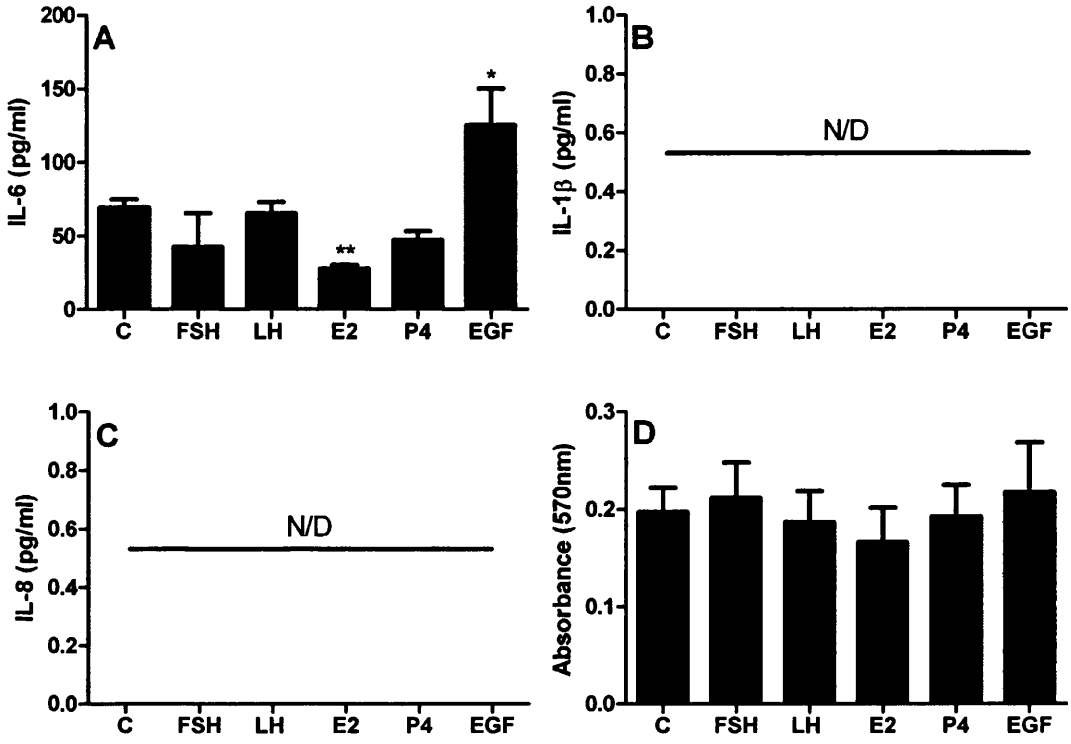


Figure 3.14 EGF increased accumulation of IL-6. Granulosa cells from emerged follicles were treated for 24 h with medium containing 100 ng/ml FSH, 4 ng/ml LH, 500 ng/ml E2, 250 ng/ml P4 or 10 ng/ml EGF. Supernatants were collected and accumulation of IL-6 (A), IL-1β (B) and IL-8 (C) measured by ELISA. Data are presented as mean + SEM and represent 4 independent experiments. Values differ from control by ANOVA and Dunnett's pairwise multiple comparison t-test (log_{10} normalised data), * $P < 0.05$, ** $P < 0.01$. (D) Cell viability was assessed by MTT test. Data presented as mean + SEM from 3 independent experiments. N/D = below limits of detection.

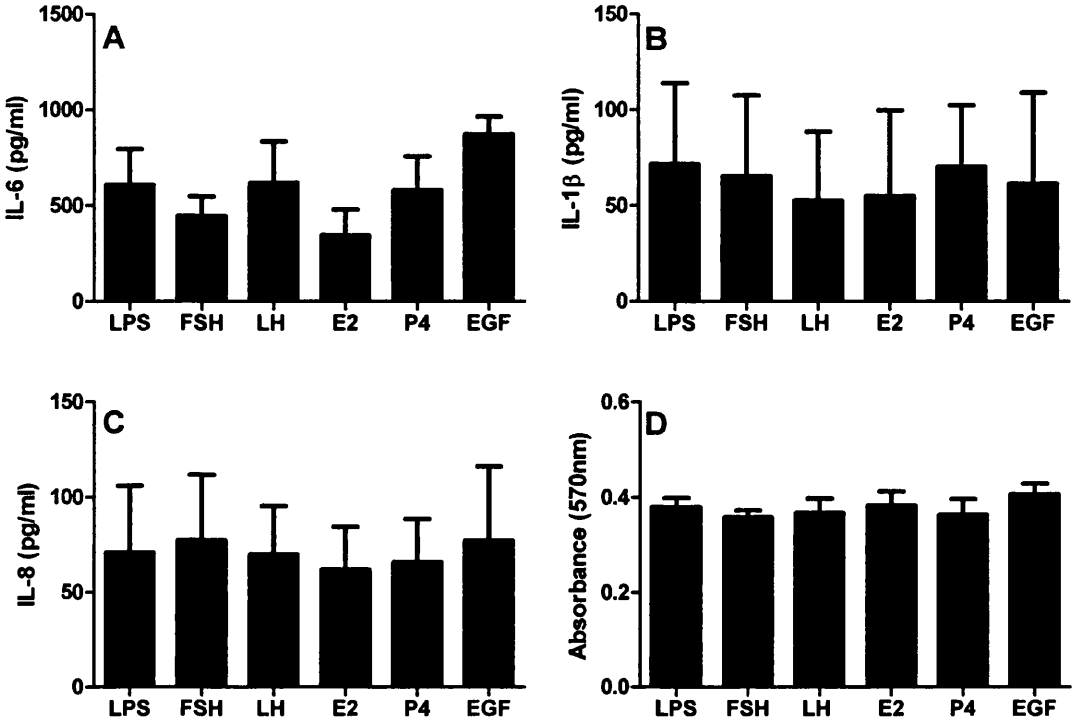


Figure 3.15 EGF had no significant effect on the accumulation of IL-6 by GCs treated with LPS. Granulosa cells from emerged follicles were treated for 24 h with medium containing 1 μg/ml LPS in combination with 100 ng/ml FSH, 4 ng/ml LH, 500 ng/ml E2, 250 ng/ml P4 or 10 ng/ml EGF. Supernatants were collected and accumulation of IL-6 (A), IL-1β (B) and IL-8 (C) measured by ELISA. Data are presented as mean + SEM and represent 4 independent experiments. D) Cell viability was assessed by MTT test. Data presented as mean + SEM from 3 independent experiments.

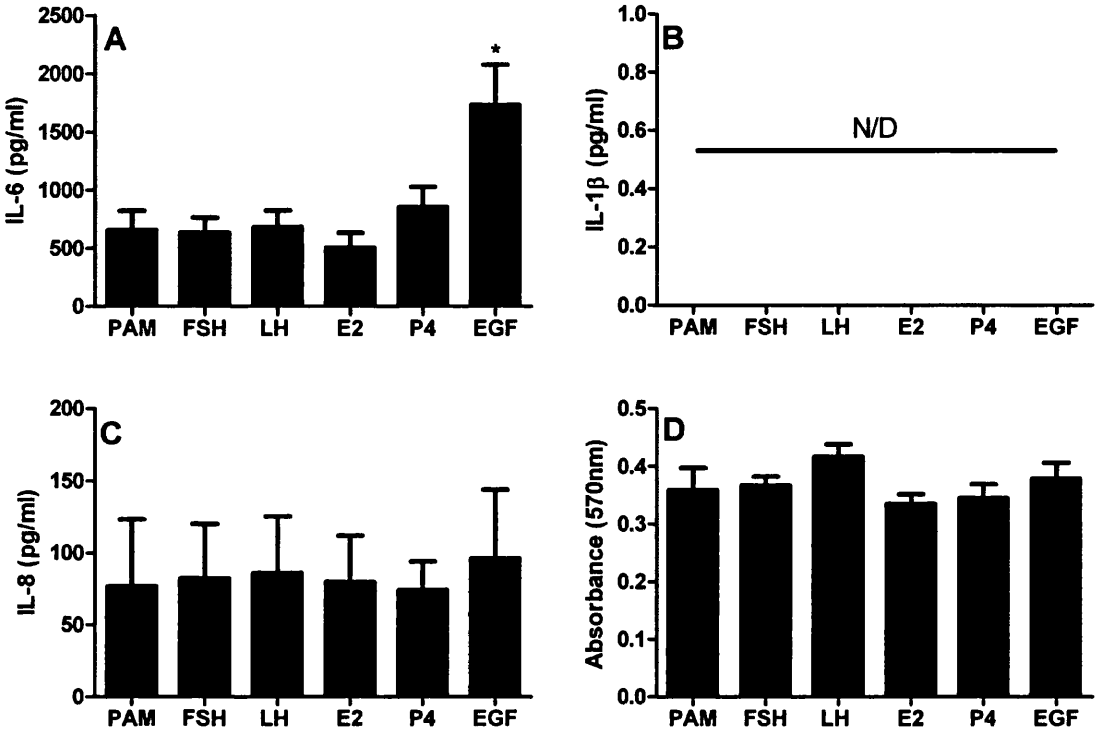


Figure 3.16 EGF increased accumulation of IL-6 by GCs treated with PAM. Granulosa cells from emerged follicles were treated for 24 h with medium containing 1 μg/ml PAM in combination with 100 ng/ml FSH, 4 ng/ml LH, 500 ng/ml E2, 250 ng/ml P4 or 10 ng/ml EGF. Supernatants were collected and accumulation of IL-6 (A), IL-1β (B) and IL-8 (C) measured by ELISA. Data are presented as mean + SEM and represent 4 independent experiments. Values differ from control by ANOVA and Dunnett's pairwise multiple comparison t-test (log_{10} normalised data), * $P < 0.05$. (D) Cell viability was assessed by MTT test. Data presented as mean + SEM from 3 independent experiments. N/D = below limits of detection.

3.3.9 Inhibiting NFκB, p38 MAPK or MEK limited LPS- and PAM-stimulated IL-6 accumulation by GCs

Granulosa cells were treated with 10-fold increasing concentrations of inhibitors targeting NFκB (range: 0.0008-80 μM), PKC (range: 0.002-200 μM), JNK (range: 0.01-1000 μM), p38 MAPK (range: 0.002-200 μM) or MEK (range: 0.002-20 μM) or DMSO vehicle (1:500) for 30 min, followed by addition of control medium or medium containing LPS (Fig. 3.17) or PAM (Fig. 3.18) to a final concentration of 1 μg/ml. Each inhibitor significantly limited the anticipated IL-6 response to LPS or PAM but also significantly decreased cell viability when used at higher doses. However, using 0.04 μM NFκB inhibitor, 1 μM MEK inhibitor or 1 – 10 μM p38 MAPK inhibitor significantly decreased LPS-stimulated IL-6 accumulation without having a significant effect on cell viability (Fig. 3.17 A, D, E). Treating GCs with 0.004 – 0.4 μM NFκB inhibitor, or 1 – 10 μM p38 MAPK inhibitor significantly limited PAM-stimulated IL-6 accumulation without significantly impacting cell viability (Fig. 3.18 A, E). To further test the use of inhibitors, inhibitors were also used in pairs at the lowest dose tested previously (0.0004 μM NFκB inhibitor, 0.001 μM PKC inhibitor, 0.005 μM JNK inhibitor, 0.0001 μM MEK inhibitor and 0.001 μM p38 MAPK inhibitor). When using most of these combinations of low concentrations of inhibitors, there was no significant effect on IL-6 accumulation or cell viability of LPS- and PAM- stimulated cells (Fig. 3.17F, 3.18F). However, low-dose MEK and JNK inhibitor combination treatment decreased the anticipated IL-6 accumulation in response to LPS without a significant effect on cell viability (Fig. 3.17F).

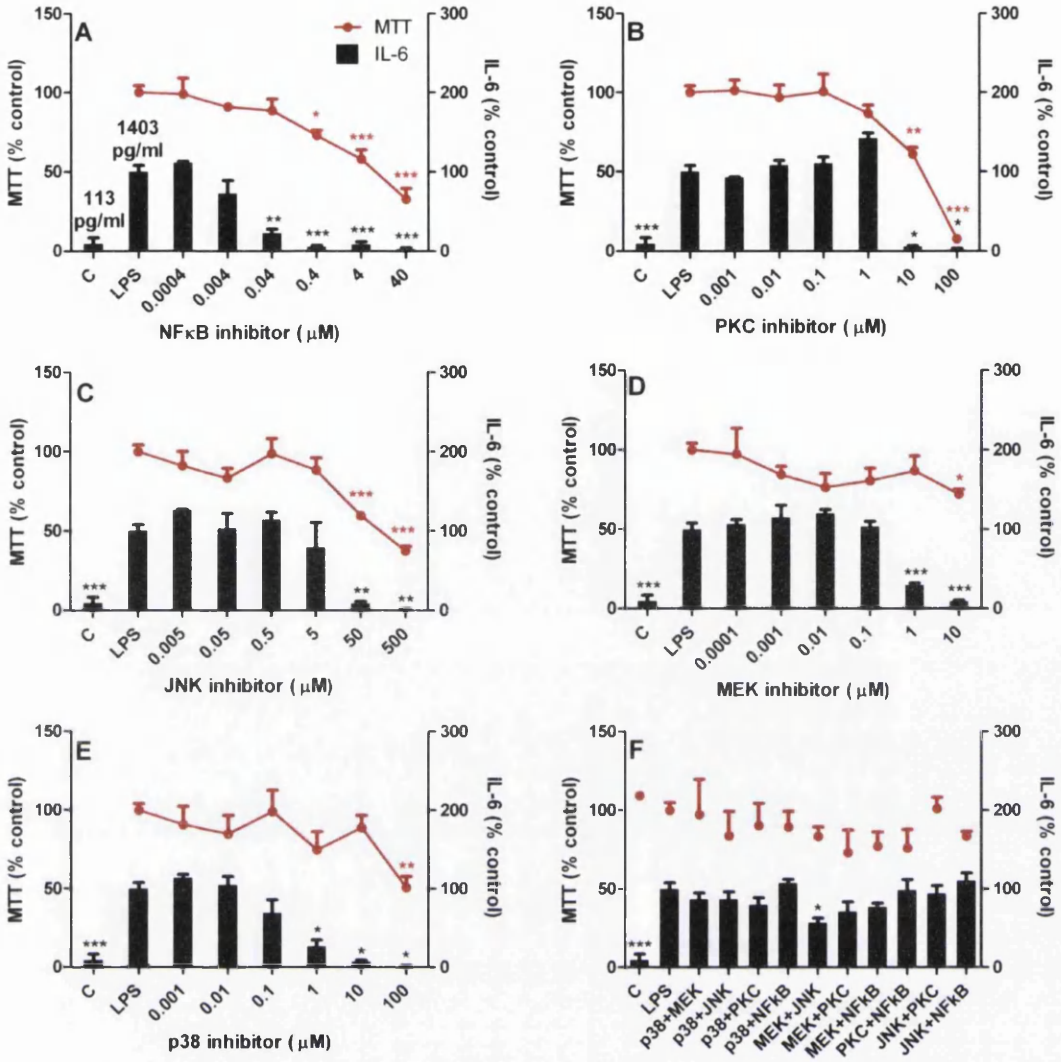


Figure 3.17 Inhibiting NFκB, MEK or p38 MAPK limited LPS-induced IL-6 accumulation by GCs. Granulosa cells from emerged follicles were treated for 30 min with 10-fold increasing concentrations of biochemical inhibitors targeting NFκB (A; 0.0004 to 40 μM), PKC (B; 0.001 to 100 μM), JNK (C; 0.005 to 500 μM), MEK (D; 0.0001 to 10 μM), p38 MAPK (E; 0.001 to 100 μM) or low dose combination treatments using pairs of inhibitors (0.0004 μM NFκB inhibitor, 0.001 μM PKC inhibitor, 0.005 μM JNK inhibitor, 0.0001 μM MEK inhibitor and 0.001 μM p38 MAPK inhibitor); followed by addition of LPS to each well making a final concentration of 1 μg/ml for 24 h. Supernatants were collected and the accumulation of IL-6 measured by ELISA (black bars). Cell viability was assessed by MTT assay (red line). Data represent mean + SEM from 3-4 independent experiments and are expressed as a percentage of LPS treatment. Values differ from LPS treatment by ANOVA and Dunnett's pairwise multiple comparison t test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

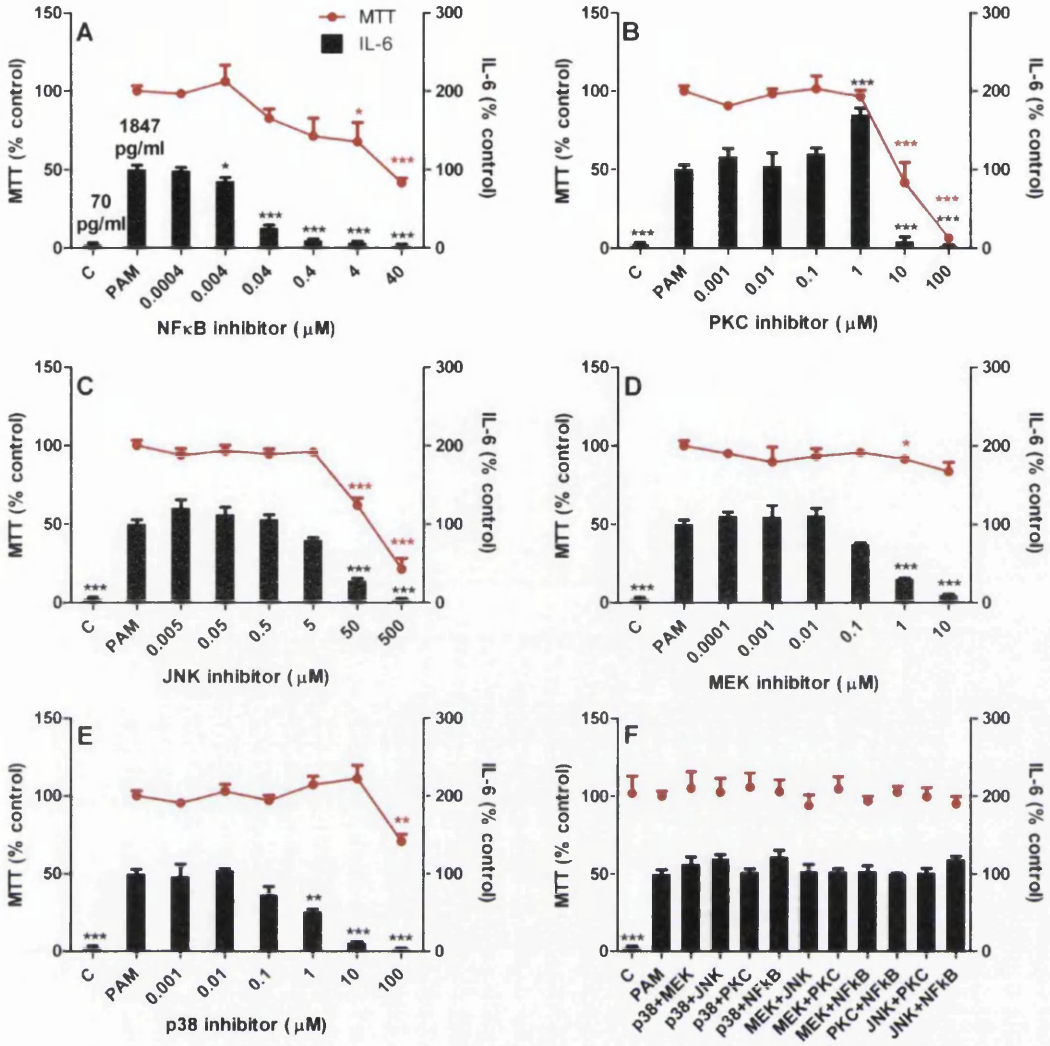


Figure 3.18 Inhibiting NFκB or p38 MAPK limited PAM-induced IL-6 accumulation by GCs. Granulosa cells from emerged follicles were treated for 30 min with 10-fold increasing concentrations of biochemical inhibitors targeting NFκB (A; 0.0004 to 40 μM), PKC (B; 0.001 to 100 μM), JNK (C; 0.005 to 500 μM), MEK (D; 0.0001 to 10 μM), p38 MAPK (E; 0.001 to 100 μM) or low dose combination treatments using pairs of inhibitors (0.0004 μM NFκB inhibitor, 0.001 μM PKC inhibitor, 0.005 μM JNK inhibitor, 0.0001 μM MEK inhibitor and 0.001 μM p38 MAPK inhibitor); followed by addition of PAM to each well making a final concentration of 1 μg/ml for 24 h. Supernatants were collected and the accumulation of IL-6 measured by ELISA (black bars). Cell viability was assessed by MTT assay (red line). Data represent mean + SEM from 3-4 independent experiments and are expressed as a percentage of PAM treatment. Values differ from PAM treatment by ANOVA and Dunnett's pairwise multiple comparison t test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.4 Discussion

Bacterial infections of the uterus or mammary gland perturb ovarian dominant follicle growth and endocrine function (Sheldon et al., 2002, Lavon et al., 2011a, Lavon et al., 2011b). Whilst LPS has been found in the follicular fluid from dominant follicles of animals with uterine disease (Herath et al., 2007), healthy ovarian follicles are devoid of immune cells (Spanel-Borowski et al., 1997, Bromfield and Sheldon, 2011). Infections of the bovine female genital tract are common and are associated with trauma during parturition (Potter et al., 2010). *Escherichia coli* is the first pathogen to invade the uterus, whose LPS collects inside ovarian follicular fluid (Herath et al., 2007). Previously, it was hypothesised that TLRs are involved in the physiological process of ovulation in mice through interaction of DAMPs, such as hyaluronic acid, with TLRs (Shimada et al., 2008). This chapter tested the hypothesis that primary bovine GCs from emerged follicles respond to PAMPs and DAMPs, by examining the response of TLRs to pathological and physical stimuli. Granulosa cells from emerged follicles expressed *TLRs 1-10* and responded to the PAMPs LPS and PAM at the protein level and to LPS, PAM and FLA at the RNA level. However, there was no significant inflammatory response to the DAMP HA. Treatment of GCs with LPS or PAM activated intracellular signals associated with TLR activation, and the IL-6 response to PAM and LPS required TLR2 and TLR4, respectively. When investigating ways to manipulate the response, it was shown that EGF may increase the accumulation of IL-6 by GCs and it was possible to limit LPS- or PAM- stimulated IL-6 accumulation using biochemical inhibitors targeting NFκB, MEK or p38 MAPK.

Toll-like receptors are conserved structures expressed mainly by cells of the immune system such as macrophages and dendritic cells (Akira et al., 2006, Takeuchi and Akira, 2010, Moresco et al., 2011). However, a wider repertoire of cells appears to have roles in innate immunity, including the epithelial cells of the intestine (Gribar et al., 2008) and endometrium (Cronin et al., 2012). A striking feature of healthy ovarian follicles is that they do not contain immune cells within the follicle (as shown in chapter 2), so GCs may play a role in innate immunity inside ovarian follicles. Previous studies have identified expression of *TLR4* in bovine GCs (Herath et al., 2007) and *TLR2* and *TLR4* in murine (Shimada et al.,

2008) and human (Woods et al., 2011) GCs. In the present study, bovine GCs from emerged follicles expressed mRNA encoding all 10 TLRs with high expression of *TLR3* compared to all other TLRs at time 0 and after 51 h culture. Although changes in gene expression may have little bearing on immune function, this high expression of *TLR3* was notable as viruses such as bovine viral diarrhoea virus infect the ovary of cattle and perturb dominant follicle growth (Grooms et al., 1998). Interestingly, treatment of GCs with LPS or PAM increased expression of *TLR2*, suggesting a potential priming effect with one PAMP enhancing the sensitivity of the GCs to another TLR2 agonist. However, when GCs were pre-treated with LPS or PAM, before a further PAM treatment, there was no additional output in IL-6. This could be because the increased mRNA expression did not result in an increase in protein, or it may be that an output other than IL-6 would have increased. Alternatively, the increased *TLR2* expression may have occurred to increase responses to non-bacterial pathogens, such as herpesvirus virions (Paludan et al., 2011).

Ideally, GCs are cultured in the absence of serum to prevent luteinisation (Gutiérrez et al., 1997). However, it was previously shown that bovine GCs do not increase expression of *IL6* mRNA or accumulate IL-6 or IL-8 in response to PAMPs in serum-free conditions (Bromfield and Sheldon, 2011). The lack of LPS response may be due to the need for soluble CD14, LY96 or LPS-binding protein. Previously, it was demonstrated that CD14, but not LY96, is required for the response of human whole blood to PAM and LPS (Elson et al., 2007) and CD14 is required for a robust response to PAM in many cellular systems (Scott and Smith, 1997, Okazaki et al., 2010). Thus, as GCs also lacked a response to TLR2 ligands in the absence of serum, it seems likely that bovine GCs require soluble CD14 to produce an inflammatory response to PAMPs, despite them expressing *CD14* mRNA.

In the previous chapter (Materials and Methods), it was shown that LPS increased expression of *IL6, IL1B* and *IL8* from 1 h and this was maintained for at least 5 h, and so 3 h was chosen as a suitable time point to examine responses to PAMPs at the RNA level. The ligands for TLR2 (PAM), TLR4 (LPS) and TLR5 (FLA) increased expression of genes encoding inflammatory mediators *IL6, IL1B, IL10, TNF, IL8* and *CCL5*. This response to LPS supports previous observations that bovine GCs from emerged follicles increased expression of *IL6* after 30 min LPS exposure and *IL8* after 90 min, with increased expression maintained for at least

180 min (Bromfield and Sheldon, 2011). A similar response is also exhibited by hen GCs with increased expression of *IL1B* after 4 h LPS treatment (Woods et al., 2009). There do not seem to be published observations using PAM or FLA in GCs from any species.

The accumulation of pro-inflammatory cytokines (IL-6, IL-1β, TNFα and IL-8) increased in a concentration-dependent manner in response to LPS and PAM, but not FLA. It is possible that this is because flagellin does not collect inside the ovarian follicle, thus there is no need for GCs to respond to flagellin, or it may be that although the receptor is expressed at the mRNA level, this is not translated into protein. Due to difficulties in sourcing bovine antibody against TLR5, it was not possible to examine the localisation of the TLR. The cellular response of bovine GCs to LPS and PAM was similar to the response previously observed, showing increased accumulation of IL-6 and IL-8 in response to LPS, PAM and to the TLR2 ligands lipoteichoic acid and peptidoglycan (PGN) (Bromfield and Sheldon, 2011). There was a significant increase in inflammatory cytokine production from 0.01 μg/ml LPS treatment, corresponding to the concentration of LPS measured (range 4.5-875 ng/ml) in the follicular fluid of cows with endometritis (Herath et al., 2007). The present work used the synthetic ligand PAM as a TLR2 agonist to ensure greater target specificity than PGN. The importance of this approach was evident because preliminary work with PGN induced accumulation of IL-6, an observation that could not be replicated when using an ultrapure form of PGN. It is possible that the PGN used initially was contaminated with other bacterial components. Also, PGN may be an agonist of nucleotide-binding oligomerisation domain-containing protein (NOD)-like receptors, rather than binding TLR2 (Inohara et al., 2003, Travassos et al., 2004).

From a pathological viewpoint, the increased chemokine production of GCs would produce a gradient attracting macrophages and neutrophils towards the ovary, helping to clear the oviduct and peritoneal cavity from bacteria prior to ovulation, thus protecting the oocyte. However, more interestingly, there may be an effect of increased cytokine production on the physiological function of the ovarian follicle. Interleukin-6 induces murine cumulus-oocyte complex (COC) expansion and improves oocyte competence (Liu et al., 2009). Thus, when a PAMP, such as LPS, is present in the follicle and induces GCs to produce IL-6, this may lead to early COC

expansion. Interleukin-1β decreases production of progesterone receptor and steroidogenic acute regulatory protein in equine cumulus cells and also has a negative impact on EGF-stimulated oocyte maturation (Caillaud and Gérard, 2009). However, during bovine *in-vitro* maturation (IVM), the addition of IL-1β to the culture medium has no effect on cleavage rate or blastocyst development (Al Naib et al., 2011). When bovine GCs from small (<5 mm) follicles were treated with IL-1β or TNFα, their FSH-stimulated oestradiol production was inhibited (Spicer and Alpizar, 1994). Oestrogen is associated with follicle growth and anti-apoptosis in GCs (Quirk et al., 2006). Thus by decreasing oestrogen concentration, the follicle may prevent an unhealthy oocyte from being ovulated and possibly forming an unhealthy embryo.

Hyaluronic acid is a major component of follicular fluid, and has been shown to act as a danger signal through TLR2 and TLR4 (Taylor et al., 2004, Scheibner et al., 2006). The present work investigated whether bovine GCs exhibit observations typical of an innate immune response after treatment with HA and found that there was no detectable response to ultra-low (ULMW) or high molecular weight (HMW) forms. This is in contrast to observations using HA with murine monocytes (Yamawaki et al., 2009), macrophages (Jiang et al., 2005) and COCs (Shimada et al., 2008). The difference in these responses may be due to purity of the DAMP used – in many previous studies *E. coli* was used as a transfection vector to obtain HA, thus it is likely that contaminating LPS may have been present. Supporting the finding in this chapter, that HMW HA limited the IL-6 accumulation in response to LPS, HA suppresses the LPS-stimulated release of IL-6 by murine macrophages (Muto et al., 2009). The finding that HA does not stimulate TLRs in the GC model to be used in the following chapters is useful, as it means we can be clear that any observations are due to treatment with PAMPs, not a response to the damage caused during follicle aspiration and GC isolation.

The present work showed that TLRs were expressed by GCs and that GCs respond to TLR ligands. In order to link these two observations, siRNA was used to target *TLR2* or *TLR4* (with 79% or 82% efficiency, respectively), and limit the IL-6 response to PAM or LPS, respectively. Targeting *TLR4* has been used previously to test the impact of LPS in bovine GCs (Bromfield and Sheldon, 2011) or endometrial cells (Cronin et al., 2012). Further confirmation of active TLR pathways in the

present study was the phosphorylation of p38 MAPK and ERK1/2 following treatment of GCs with LPS or PAM. The kinetics of phospho-p38 MAPK and phospo-ERK1/2 in GCs in this work was similar to those published in macrophages, with rapid phosphorylation of both signals induced by PAMP treatment and maintained for at least 20 minutes (Feng et al., 1999, Kong and Ge, 2008). Activation of these MAPKs leads to increased expression and secretion of pro-inflammatory cytokines. Conversely, inhibiting these pathways decreases the IL-6 accumulation in response to LPS or PAM, as shown in this chapter using biochemical inhibitors for p38 MAPK and MEK. This work on inhibitors pointed to 3 pathways worth further investigation: NFκB, p38 MAPK and MEK, which are pathways also shown to be interesting in a study using computational modelling to investigate anti-inflammatory combinations of small molecule inhibitors aimed at decreasing expression of *IL1B* (Small et al., 2011). Due to the detrimental effects of inhibiting key intracellular signals on cell viability, and the increased interest in combination therapy, very low concentrations of inhibitors were used in pairs, at concentrations which had no detrimental effect on cell viability or IL-6 accumulation when used singularly. Interestingly, when using inhibitors targeting MEK and JNK in combination, the accumulation of LPS-stimulated IL-6 was decreased. This pair points to a possible therapeutic combination which may warrant further investigation identifying the optimum concentration of each inhibitor, or trying different inhibitors targeting the same pathway.

From above, we can see that the pathological response to PAMPs may affect the physiological role of the ovary. However, it is also possible that physiological actions may affect the pathological response. The stage of oestrous cycle affects the accumulation of IL-6, IL-1β and IL-8 by bovine uterine explants in response to LPS (Borges et al., 2012), suggesting that hormones may crosstalk with innate immune responses. In this chapter, it was reported that EGF, which helps to coordinate LH-mediated events including oocyte maturation and COC expansion (Conti et al., 2006), increased accumulation of IL-6, but not IL-8 or IL-1β by GCs treated with control medium or medium containing PAM. This is consistent with results using ovarian epithelial cancer cell lines, which showed increased *IL6* expression after EGF treatment through STAT3 and JAK2 (Colomiere et al., 2008) and increased expression after treatment with an EGFR ligand through NFκB (Alberti et al., 2011).

It is unsurprising that LH had no effect on cytokine accumulation by GCs from emerged follicles, because GCs only express the LH receptor after a follicle reaches dominance. Treatment of GCs with PAM increased expression of *EGFR* within 3 h. Previously, it was shown that TNFα up-regulated *EGFR* expression in human colonic myofibroblasts (Yoo et al., 2012). However, in the present thesis, both PAM and LPS lead to increased TNFα accumulation only at 10 μg/ml, and LPS did not affect *EGFR* expression, thus it seems unlikely that this is the mechanism which causes increased *EGFR* expression in response to PAM. In epithelial cells, agonists of TLR2 increase activation of EGFR, although expression of *EGFR* was not examined in this study (Shaykhiev et al., 2008). The EGF receptor negatively regulated bacteria-induced TLR2 expression in HeLa cells (Mikami et al., 2005). It seems possible that if EGFR negatively regulates *TLR2* signalling, increased TLR2 signalling (through PAM) may cause an increase in *EGFR* expression as a negative feedback loop to control the response.

A previous study found that FSH treatment increased accumulation of IL-8 by GCs from emerged follicles after 24 h or 48 h and increased IL-6 accumulation after 48 h (Bromfield and Sheldon, 2011). In contrast, the present study found no significant effect of FSH on accumulation of LPS-stimulated cytokine production. These contrasting results are likely due to the different concentration of FSH used (although the concentration of FSH used in this project was supraphysiological at 100 ng/ml, it was lower than the concentration used in the 2011 study of 2.5 μg/ml). Follicle-stimulating hormone (at 31-2000 ng/ml) also increased IL-6 accumulation by rat GCs at the 48 h time point (Gorospe and Spangelo, 1993), suggesting that it may be interesting to extend the present work to study a wider range of temporal responses.

In the present work, there was no significant effect of oestradiol or progesterone on LPS- or PAM-stimulated IL-6 accumulation, although oestradiol significantly decreased IL-6 accumulation by cells in control medium. This is in contrast to the effect of progesterone on foetoplacental arteries (Shields et al., 2005) and the observation that human peripheral blood monocytes isolated during the luteal phase (when progesterone concentrations are higher) produced less IL-6 (Schwarz et al., 2000). Similarly, oestrogen decreased accumulation of IL-6 by peripheral blood mononuclear cells (Rachon et al., 2002) and down-regulated the IL-6 promoter in the

HeLa cell line (Delrieu et al., 2000). Although these results support the findings in this chapter, it seems strange that increased oestradiol (as seen just prior to ovulation) decreases IL-6 accumulation, which seems to play a role in oocyte maturation and cumulus expansion, which needs to occur prior to ovulation. Potentially, the increased oestradiol acts as a brake in oocyte maturation by regulating the concentration of IL-6 in the follicle.

In conclusion, GCs from emerged follicles expressed TLRs and produced a cellular response characteristic of a TLR2 and TLR4 innate immune response. Treatment with PAM or LPS increased accumulation of inflammatory mediators IL-6, IL-1β, TNFα and IL-8, and increased phosphorylation of p38 MAPK and ERK1/2. The increased accumulation of IL-6 in response to PAMPs was decreased using biochemical inhibitors targeting p38 MAPK, NFκB or MEK, highlighting the importance of these signals in the TLR cascade. Surprisingly, the DAMP HA had no significant effect on GCs, although modulated the IL-6 response to LPS, with a high concentration of the HMW form limiting the IL-6 accumulation. The expression of *EGFR* and *FSHR* was not affected by PAMP treatment, but EGF significantly increased accumulation of IL-6, providing evidence for crosstalk between immune and endocrine pathways. Overall, GCs from emerged follicles may have roles in innate immunity, at least against pathogens.

Chapter 4

Pathogen-associated molecular patterns initiated inflammation and perturbed the endocrine function of granulosa cells from dominant follicles via TLR2 and TLR4 pathways

4.1 Introduction

In the previous chapter, the interactions between pathogen-associated molecular patterns (PAMPs) and granulosa cells (GCs) from emerged ovarian follicles were investigated. Emerged follicles are a homogeneous population, most of which will become atretic, with usually only a single follicle progressing to dominance. In this chapter, GCs from dominant follicles will be used, which differ from those in emerged follicles, as these will mainly come from follicles which have the potential to ovulate. *In vivo* observations show that uterine disease has the greatest impact on dominant follicles, with reduced growth rate and steroidogenesis (Sheldon et al., 2002, Williams et al., 2007). Thus, although the *in vivo* observations may be due to the effect of infection on growing emerged follicles, investigating the impact of infection on dominant follicles may be more biologically relevant than experiments using smaller follicles.

In cattle, a single dominant follicle is usually selected from a cohort of smaller emerged follicles that develop in waves in response to an increased concentration of FSH in peripheral plasma (Adams et al., 1992). Uniquely amongst ovarian follicles, the dominant follicle may go on to ovulate, rather than undergoing atresia. Interestingly, any viable emerged follicle is capable of becoming dominant, as cows can be super-ovulated with FSH (Adams et al., 1993, Ireland et al., 2007); one randomly selected 5 mm follicle can be directed to become dominant if all other follicles of a similar size are destroyed (Gibbons et al., 1997), and the subordinate follicle will become dominant if the dominant follicle is ablated prior to day 15 of the oestrous cycle (Dean and Dailey, 2011).

The future dominant follicle emerges 7 h earlier than the largest subordinate follicle, probably because it has a small developmental advantage over the other follicles, such as being closer to the ovarian vasculature or having more cells (Kulick et al., 1999). Prior to deviation, all follicles grow at the same rate (Ginther et al., 1997). However, because the future dominant follicle emerged first, it is larger and thus maintains its advantage over the other follicles in the cohort, because the rate of growth of the surface area of a spherical object is greater for the sphere with the larger radius. Thus, the larger follicle increases its surface area at a greater rate, reflected by more GCs within the follicle and a greater number of FSH receptors. As

FSH concentrations decline, the larger follicle is still able to grow due to its increased number of FSH receptors. The greater response to FSH in the larger follicle also increases the production of insulin-like growth factor (IGF) binding protein (IGFBP) proteases (Rivera and Fortune, 2001), which cleave IGFBPs, increasing the concentration of bio-available IGF-1 within the follicle (Rivera and Fortune, 2003). The bio-available IGF-1 acts synergistically with FSH to increase the production of oestradiol by the dominant follicle (Adashi, 1998), which then suppresses FSH thus starving the smaller follicles of their gonadotrophin drive (Burke et al., 1996, Ginther et al., 2000). It has also been hypothesised that other substances produced by the dominant follicle may suppress FSH including inhibin (Lussier et al., 1993, Kaneko et al., 1997), which may be synthesised by the dominant follicle in response to falling concentrations of FSH, thus further suppressing the subordinate follicles (Mihm et al., 1997).

During follicle selection, the GCs within the dominant follicle start to express mRNA encoding the LH receptor (Xu et al., 1995, Nogueira et al., 2007). This enables the follicle to grow independently of FSH, thus as FSH concentrations decline, subordinate follicles without LH receptors are unable to grow or develop and undergo atresia, whilst the dominant follicle continues to grow under the influence of LH. If LH pulse frequency remains low, the dominant follicle undergoes atresia, FSH is no longer suppressed and a new wave begins (Roche, 1996). If LH pulse frequency increases and circulating concentrations of progesterone are low, the dominant follicle persists until ovulation. After ovulation, the GCs begin to luteinise and increase production of progesterone, forming the corpus luteum. A number of processes require LH including GC luteinisation, cumulus expansion and oocyte maturation (Yamashita and Shimada, 2012, Sayasith et al., 2013). However, LH receptors are mainly expressed on GCs and not on cumulus cells or oocytes (Peng et al., 1991). It is thus hypothesised that LH signals are propagated through the follicle by EGF-like factors, such as epiregulin and amphiregulin (Park et al., 2004, Ashkenazi et al., 2005, Jamnongjit et al., 2005). A recent paper supporting this theory reported that cows treated with gonadotrophin increase granulosa expression of epiregulin and amphiregulin and bovine GCs treated with EGF increase expression of mRNA encoding genes related to ovulation (Sayasith et al., 2013).

The characteristics of GCs within the dominant follicle include increased expression of *CYP19A1*, increased conversion of androstenedione to oestradiol, and expression of luteinising hormone receptors (*LHCGR*) in addition to their existent FSH receptors (*FSHR*) (Fortune et al., 2001). Granulosa cell function not only regulates dominant follicle growth and function, but also directs ovulation of the oocyte, with ovulation triggered by LH but mediated by EGF that induces a cascade of events resembling inflammation (Matzuk et al., 2002, Park et al., 2004).

In vivo, the impact of uterine infection on ovarian function is only detectable on dominant ovarian follicles, with decreased follicle growth rate and reduced oestradiol production (Sheldon et al., 2002, Williams et al., 2007). In addition, LPS was measured in the follicular fluid from dominant, not emerged, ovarian follicles (Herath et al., 2007). Previous studies have focussed on GCs from emerged follicles, hypothesising that the damage to the follicles only becomes apparent after attaining dominance, but that the damage was caused whilst follicles were smaller (Bromfield and Sheldon, 2011). Although this is not improbable, it seems likely that uterine infection may also impact dominant follicle health directly.

The hypothesis for this chapter was that GCs from dominant follicles respond to pathogen-associated molecular patterns and that this response is influenced by endocrine factors.

The aims of this chapter were to investigate the innate immune response of GCs from dominant follicles to three PAMPs (LPS, PAM and FLA), and to explore the interactions between ovarian physiology (the endocrine pathway) and pathology (the innate immune response). Firstly, expression and accumulation of pro-inflammatory cytokines and chemokines was measured after PAMP exposure by qPCR and ELISA, respectively. Activation of TLR signalling components was identified using Western blot and the effect of blocking the TLR pathway was investigated using short interfering RNA (siRNA) and biochemical inhibitors. The effect of LPS and PAM on expression of endocrine receptors and production of oestradiol and progesterone was then identified, followed by the effect of endocrine factors on PAMP-stimulated pro-inflammatory cytokine production.

4.2 Methods

4.2.1 Granulosa cell culture

To explore the impact of PAMPs, GCs from dominant (> 8.5 mm) follicles from beef cows were isolated and plated in 12-, 24- or 96-well plates at a density of $1.5x10^6$ cells/ml in culture medium, as described in chapter 2. Granulosa cells from beef cattle represent a suitable model for investigating the effects of infection and inflammation on dairy GCs (chapter 2). Furthermore, GCs from follicles > 8.5 mm in diameter represented GCs from dominant follicles, with expression of *FSHR* and *LHCGR* and high expression of *CYP19A1* (chapter 2). The GC cultures were also free from contaminating immune cells, thus validating follicle aspiration as a suitable method for GC isolation (chapter 2).

After 48 h, the GCs were washed in PBS, then treated with control medium or medium containing ultrapure lipopolysaccharide from *Escherichia coli* O111:B4 (LPS; Invivogen), Pam3CSK4 (PAM; Invivogen) or flagellin from *Salmonella typhimurium* (FLA; Invivogen). Cellular responses of GCs were tested using 10-fold increasing concentrations from 0.001 to 10 μg/ml LPS or PAM and 0.0001 to 1μg/ml FLA. In subsequent experiments using PAMPs, GCs were challenged with 1 μg/ml LPS, 1 μg/ml PAM or 0.1 μg/ml FLA for 3 or 24 h for analysis by qPCR or ELISA, respectively; or for up to 5 to 25 min for analysis by Western blot.

To investigate the interaction between endocrine and immune pathways, GCs were treated with 100 ng/ml FSH (Henderson et al., 1982, McLaughlin et al., 2010), 4 ng/ml LH (Henderson et al., 1982, Fortune and Hansel, 1985), 500 ng/ml oestradiol (E2) (Ginther et al., 2001, Beg et al., 2002), 250 ng/ml progesterone (P4) (Martin et al., 1991, Ginther et al., 2001) or 10 ng/ml epidermal-like growth factor (EGF) (Langhout et al., 1991, Park et al., 1997) in combination with 1 μg/ml LPS or PAM. At the end of each experiment, supernatants were collected and stored at -20°C prior to protein analysis by ELISA; cells were washed with PBS and an MTT test carried out to estimate cell viability, as described in chapter 2. For experiments where the method of analysis was qPCR or Western blot, cells were washed twice with PBS and then lysed using 350 μl buffer RLT or 80 μl Phosphosafe, respectively.

4.2.2 Chemical inhibitors

To explore the role of intracellular signalling pathways in the response of GCs to PAMPs, GCs were seeded in a 96-well plate (TPP), equilibrated for 48 h and washed with PBS. The GCs were then treated for 30 min with 50 µl of medium containing a range of concentrations of inhibitors targeting NFκB (0.0008-80 µM), PKC (0.002-200 µM), JNK (0.01-1000 µM), p38 MAPK (0.002-200 µM) or MEK (0.002-20 µM; all Calbiochem) or DMSO (1:500). This 30 minute treatment was followed by addition of 50 µl control medium, or 50µl medium containing 2 µg/ml LPS or 2 µg/ml PAM. In a further experiment, to limit the impact of inhibitors on cell health, inhibitors were also used at their lowest concentration in combination with each other using pairs of 25 µl inhibitors targeting NFκB (0.0016 µM), PKC (0.004 µM), JNK (0.02 µM), p38 MAPK (0.004 µM) or MEK (0.004 µM) for 30 min, followed by 50 µl of medium containing LPS or PAM treatment as above.

4.2.3 Short-interfering RNA

To explore the role of TLRs, GCs were transfected with short-interfering RNA targeting *TLR2* or *TLR4* (as described in chapter 2). After 24 h, cells were washed with PBS and lysed using buffer RLT for analysis of TLR expression by qPCR, or treated with control medium or medium containing 1 µg/ml LPS or PAM for 24 h. Supernatants were collected after 24 h and stored at -20°C for analysis by ELISA; cell viability was assessed by MTT assay, as described in chapter 2.

4.2.4 ELISA

Accumulation of IL-6, IL-8, IL-1β and TNFα was measured in the supernatant of GCs treated with LPS, PAM or FLA, as described in general methods (chapter 2), using kits from R&D Systems for IL-8 and TNFα and kits from Thermo Fisher Scientific for IL-6 and IL-1β. Accumulation of oestradiol and progesterone was measured in the supernatant of GCs using ELISA kits from DRG Diagnostics, as described in chapter 2. Two technical replicates were used for each sample in each ELISA, from at least 3 experiments.

4.2.5 RNA extraction, cDNA synthesis and PCR

Granulosa cells were treated with control medium or medium containing 1 μg/ml LPS, 1 μg/ml PAM or 0.1 μg/ml FLA for 3 h. Cells were washed twice in PBS, lysed using 600 μl buffer RLT and scraped using a cell scraper. Total RNA was extracted using the RNeasy Mini kit, as described in general methods. Total RNA was quantified using a Nanodrop spectrophotometer and 1 μg total RNA used to synthesise cDNA, using the QuantiTect kit, as described in general methods. Expression of mRNA encoding genes considered as markers of an innate immune response (*IL6, IL1B, TNF, IL10, IL8, CXCL5*) and genes involved in the endocrine function of GCs (*FSHR, LHCGR, EGFR, CYP19A1*) was measured by qPCR, using 3 technical replicates for each sample from 3-4 experiments.

4.2.6 Western blot

In order to investigate the activation of ERK1/2 and p38 MAPK, GCs were plated in a 12-well plate, equilibrated for 24 h, washed twice with PBS and cultured in Optimem (low serum medium) for 24 h. They were then treated for 0, 5, 10, 15, 20 or 25 min with control Optimem medium, or Optimem containing 1 μg/ml LPS or 1 μg/ml PAM. The medium was discarded before the GCs were washed with PBS and 80 μl Phosphosafe Extraction Reagent (Novagen) used to lyse each well of cells. Protein was extracted and quantified using the DC assay (chapter 2), before blotting for diphosphorylated ERK1/2 (1:1000; Sigma 8159) or phosphorylated p38 MAPK (1:1000; Acris Antibodies APO5898PU-N). After imaging each blot using the ChemiDoc XRS System, gels were stripped for 7 min in Restore Western Blot Stripping Buffer and then re-stained for β-actin (1:1000; Abcam ab8226), as described in general methods.

Images of each blot were analysed using the ChemiDoc XRS System to measure peak density of each band, after adjusting for background, as described in chapter 2. Blots from 3 experiments were quantified, with 1 image shown as a representative in the results section.

4.2.7 Statistical analyses

Data are presented as mean + SEM, with statistical analyses performed using SPSS (version 16, SPSS Inc) and significance ascribed when $P < 0.05$. Analysis of variance was used to examine normally distributed data, following square root or log transformation where indicated in Results, as necessary. Dunnett's pairwise multiple comparison T-test was used to compare treatments with control. Data that is not significantly different by ANOVA are denoted with the same letter. Where data were not normally distributed as indicated in Results, comparisons between treatment and control were made using Mann Whitney U Test.

4.3 Results

4.3.1 Granulosa cells expressed mRNA for TLRs

The GCs from dominant follicles were negative for MHCII by FACS (chapter 2), indicating they were not contaminated by immune cells. Using these cells, the expression of TLRs was analysed by qPCR and the efficiencies of all primers were 100% ± 10%. All 10 TLRs were expressed by GCs, with higher expression of *TLR1, TLR3, TLR5* and *TLR6* and lower expression of *TLR8* compared to the other TLRs ($P < 0.01$; Fig 4.1A). Mononuclear cells were used as a positive control; the expression of each TLR was not significantly different to any other TLR (Fig 4.1B; $P > 0.23$).

In order to investigate whether treatment of GCs with one PAMP primed the cell to respond to another PAMP, we examined *TLR* mRNA expression after the cells were treated with LPS or PAM. Treatment of GCs for 3 h with LPS increased expression of mRNA encoding *TLR2* and decreased expression of *TLR4, TLR6, TLR7* and *TLR8.* Treatment of GCs for 3h with PAM also increased expression of mRNA encoding *TLR2* and decreased expression of *TLR6, TLR7, TLR8* and *TLR9* (Fig. 4.2A). To examine whether this change in mRNA levels corresponded to a functional change at the protein level, GCs were treated for 3 h with control medium or medium containing LPS or PAM. They were then washed twice with PBS, before culturing for a further 24 h with control medium or medium containing LPS or PAM. Interestingly, pre-treating with LPS or PAM increased accumulation of IL-6 by GCs subsequently treated with PAM for 24 h, mirroring the 3-fold increase in *TLR2* expression (Fig. 4.2B).

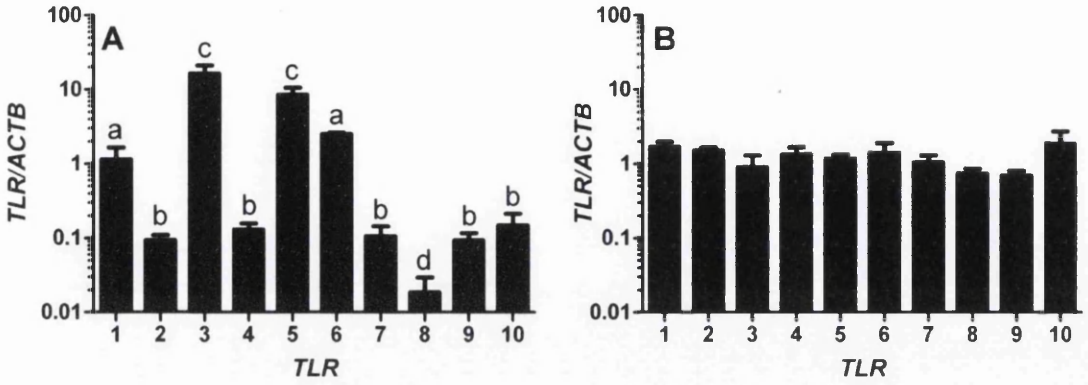


Figure 4.1 Granulosa cells expressed *TLRs 1-10*. Granulosa cells were isolated from dominant follicles (A) and bovine peripheral blood mononuclear cells (B). RNA was extracted and expression of TLRs measured by qPCR. Data represented as mean + SEM, normalised to *ACTB* and represent 3 independent experiments. Primer efficiencies were 100% ± 10% for each primer pair. Data that is not significantly different by ANOVA and post-hoc Fisher's least significant difference test (using $\log_{10}$ normalised data) are denoted with the same letter.

A

TLR	LPS		PAM	
	Fold	P	Fold	P
TLR1	0.90±0.13		0.81±0.08	
TLR2	3.06±0.79	*	3.50±1.06	**
TLR3	2.01±0.72		1.07±0.17	
TLR4	0.59±0.05	**	0.81±0.15	
TLR5	1.54±0.29		0.70±0.20	
TLR6	0.65±0.07	*	0.64±0.09	*
TLR7	0.33±0.03	***	0.34±0.06	**
TLR8	0.24±0.07	**	0.30±0.10	*
TLR9	0.83±0.09		0.56±0.06	**
TLR10	0.76±0.01	**	0.96±0.24	

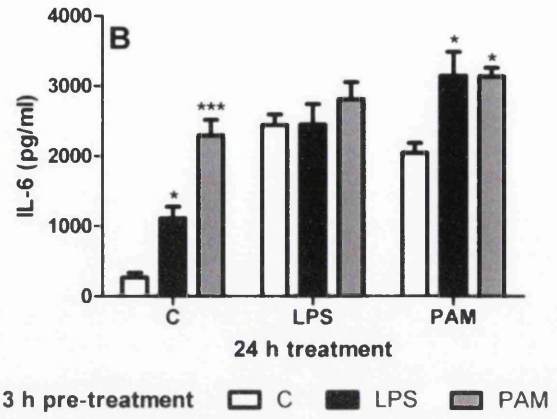


Figure 4.2 Pre-treatment of GCs with LPS or PAM impacted TLR expression. (A) Granulosa cells were treated for 3 h with control medium or medium containing 1 μg/ml LPS or PAM. The expression of mRNA encoding TLRs 1-10 was measured by qPCR and is expressed as fold change over control, relative to *ACTB*. Data presented as mean + SEM and represent 3 independent experiments. Values differ from control by Student's T-test using $\log_{10}$-normalised data; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. In a subsequent experiment, GCs were pre-treated for 3 h (as above), then further treated for 24 h with control medium ('C') or medium containing 1 μg/ml LPS or PAM. Supernatants were collected and the accumulation of IL-6 measured by ELISA (B). Data presented as mean + SEM and represent 4 independent experiments. Values differ from cells pre-treated with control medium within subsequent treatment group by ANOVA; $P < 0.05$.

4.3.2 Granulosa cells responded to PAMPs

When GCs were treated for 24 h with control medium or medium containing 10-fold increasing concentrations of LPS or PAM, supernatants accumulated IL-6, IL-1β, IL-8 and TNFα. Treatment with LPS significantly increased accumulation of IL-6 at concentrations ≥ 0.01 μg/ml (Fig. 4.3A) and increased accumulation of IL-8 and IL-1β even from the lowest concentration tested (Fig. 4.3B, D). The GCs exhibited a similar response to PAM, with increased accumulation of IL-6 from 1 μg/ml (Fig. 4.4A) and increased accumulation of IL-8 at concentrations ≥ 0.01 μg/ml (Fig. 4.4D). Interestingly, although the accumulation of IL-6 was a similar concentration whether cells were treated with LPS or PAM, treatment with PAM resulted in a lower concentration of IL-1β and a higher concentration of IL-8 than treatment with LPS.

Treatment with FLA did not increase accumulation of IL-6, IL-1β or IL-8 (all below limits of detection). No treatment increased accumulation of TNFα. For future experiments, 1 μg/ml LPS or PAM were used as the standard working concentrations because 1 μg/ml stimulated the greatest accumulation of IL-6, and 1 μg/ml LPS is similar to concentrations of LPS previously measured in the follicular fluid of cows with severe uterine disease (Herath et al., 2007).

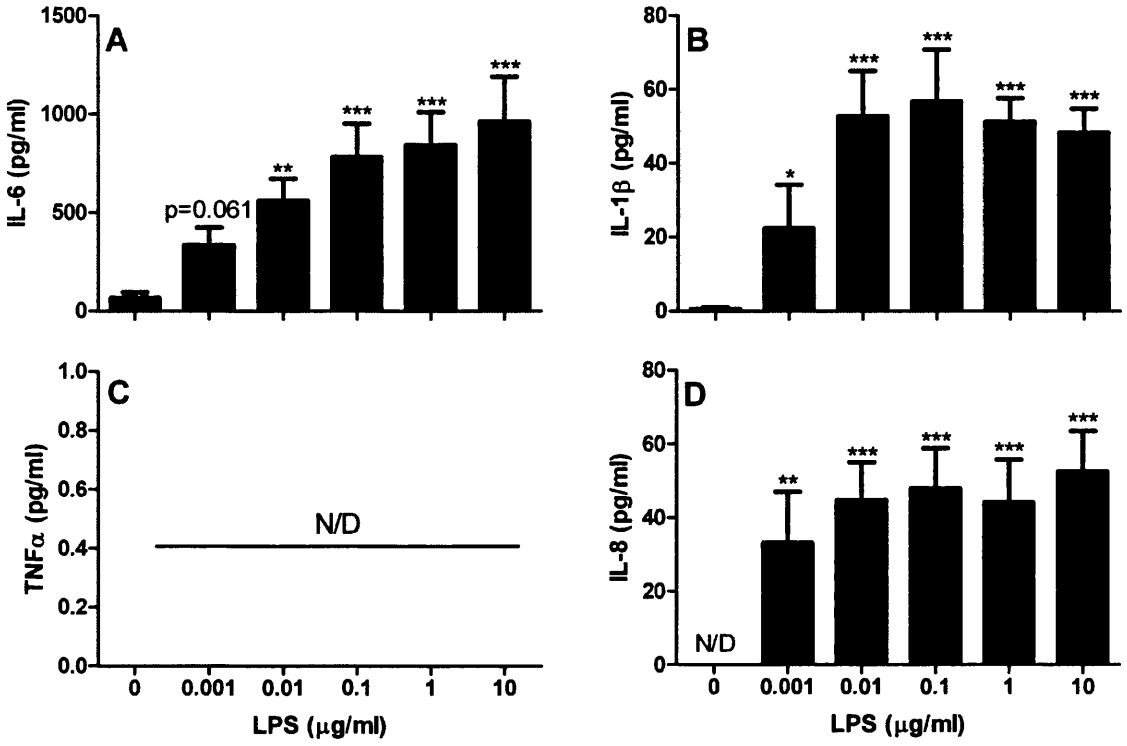


Figure 4.3 Granulosa cells responded to LPS in a concentration-dependent manner. Granulosa cells from dominant follicles were treated for 24 h with control medium ('0') or medium containing 0.001, 0.01, 0.1, 1 or 10 μg/ml ultrapure LPS. Supernatants were collected and the accumulation of IL-6 (A), IL-1β (B), TNFα (C) and IL-8 (D) measured by ELISA. Data presented as mean + SEM and represent at least 4 independent experiments. Values differ from control by ANOVA and Dunnett's pairwise multiple comparison t test (square root normalised data) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N/D = below limits of detection.

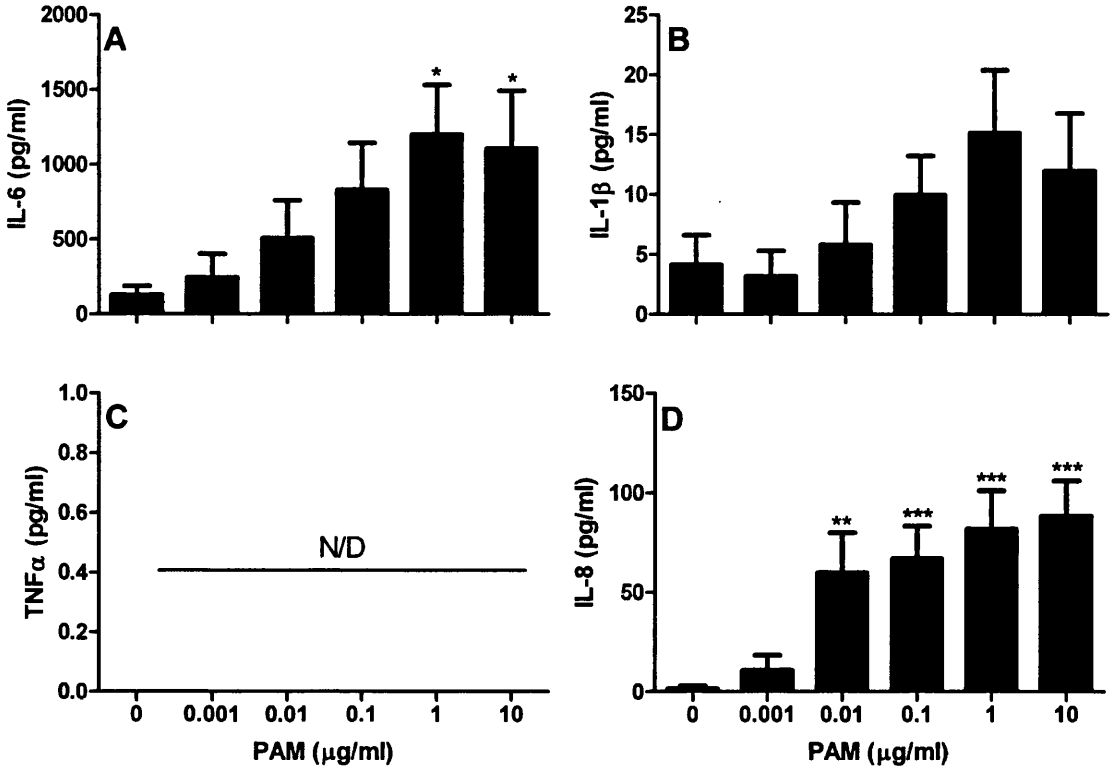


Figure 4.4 Granulosa cells responded to PAM in a concentration-dependent manner. Granulosa cells from dominant follicles were treated for 24 h with control medium ('0') or medium containing 0.001, 0.01, 0.1, 1 or 10 μg/ml Pam3CSK4 (PAM). Supernatants were collected and the accumulation of IL-6 (A), IL-1β (B), TNFα (C) and IL-8 (D) measured by ELISA. Data presented as mean + SEM and represent at least 4 independent experiments. Values differ from control by ANOVA and Dunnett's pairwise multiple comparison t test (square root normalised data) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N/D = below limits of detection.

4.3.3 Exposure of GCs to PAMPs increased expression of genes associated with inflammatory or innate immune pathways

When GCs were treated for 3 h with control medium or medium containing LPS (Fig. 4.5A-F) or PAM (Fig. 4.6A-F), they increased expression of *IL6, IL1B, IL10, TNF, IL8* and *CCL5*. All 6 of these genes associated with inflammatory or innate immune pathways had similar fold-increase for each replicate (Fig. 4.5, 4.6; each line represents one experiment, with the same colour points representing the same experiment across figures 4.5-4.6). In general, experimental replicates with high expression of one cytokine also had high expression of all other cytokines measured. Treatment with PAM increased expression of *IL1B* ($P < 0.05$), *TNF* ($P < 0.05$) and *IL8* ($P < 0.001$) to a greater extent than LPS treatment (Fig. 4.7). There was no significant effect of LPS ($P = 0.958$) or PAM ($P = 0.362$) on *ACTB* expression.

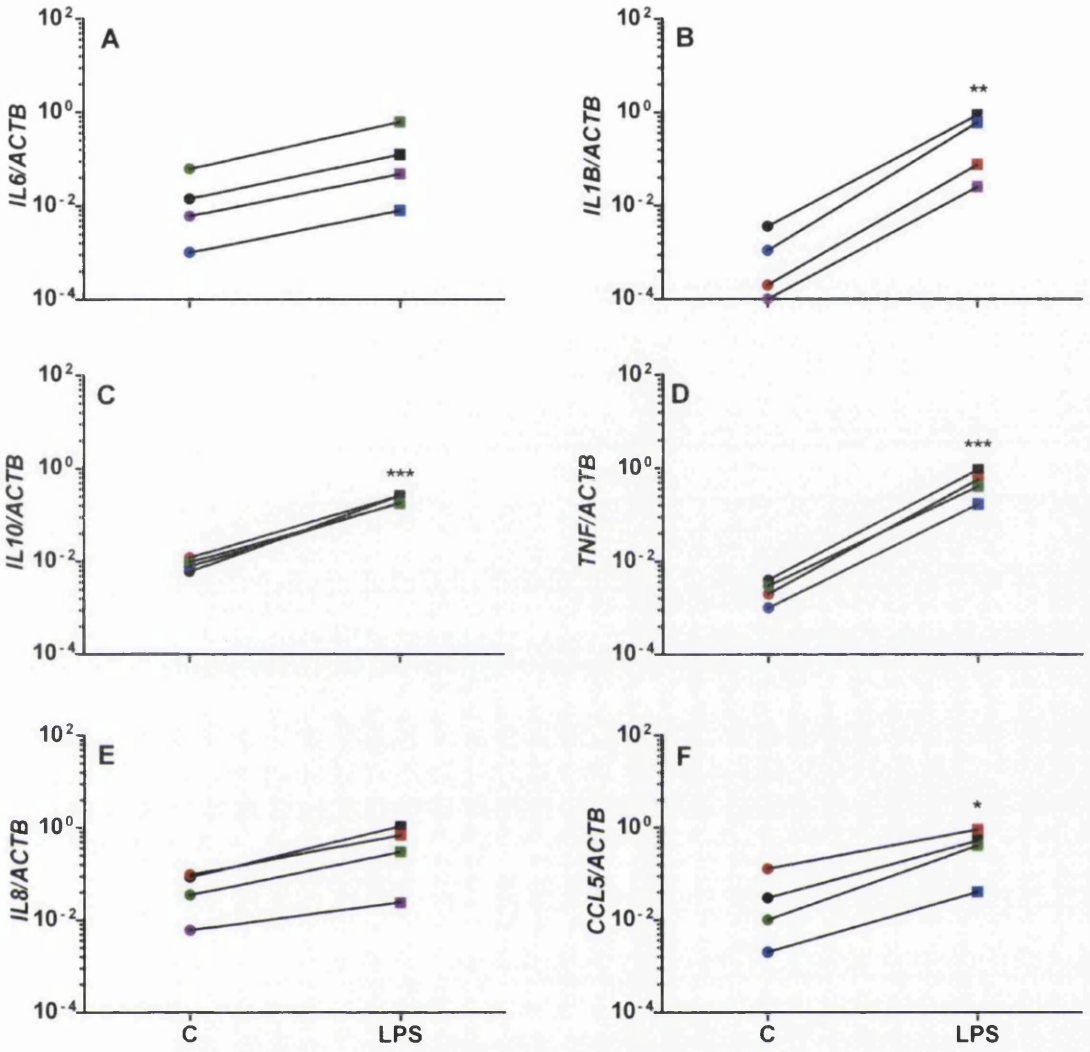


Figure 4.5 Exposure of GCs to LPS increased expression of genes associated with inflammation and innate immunity. Granulosa cells from dominant follicles were treated for 3 h with control medium ('C') or medium containing 1 μg/ml ultrapure lipopolysaccharide from *E. coli* 0111:B4 (LPS). RNA was extracted, cDNA synthesised and the expression of *IL6* (A), *IL1B* (B), *IL10* (C), *TNF* (D), *IL8* (E) and *CCL5* (F) estimated by qPCR. Each independent experiment is presented as one line, with coloured dots representing the same experiment throughout. Mean values for LPS treatment differ from control by T-test using log-normalised data, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

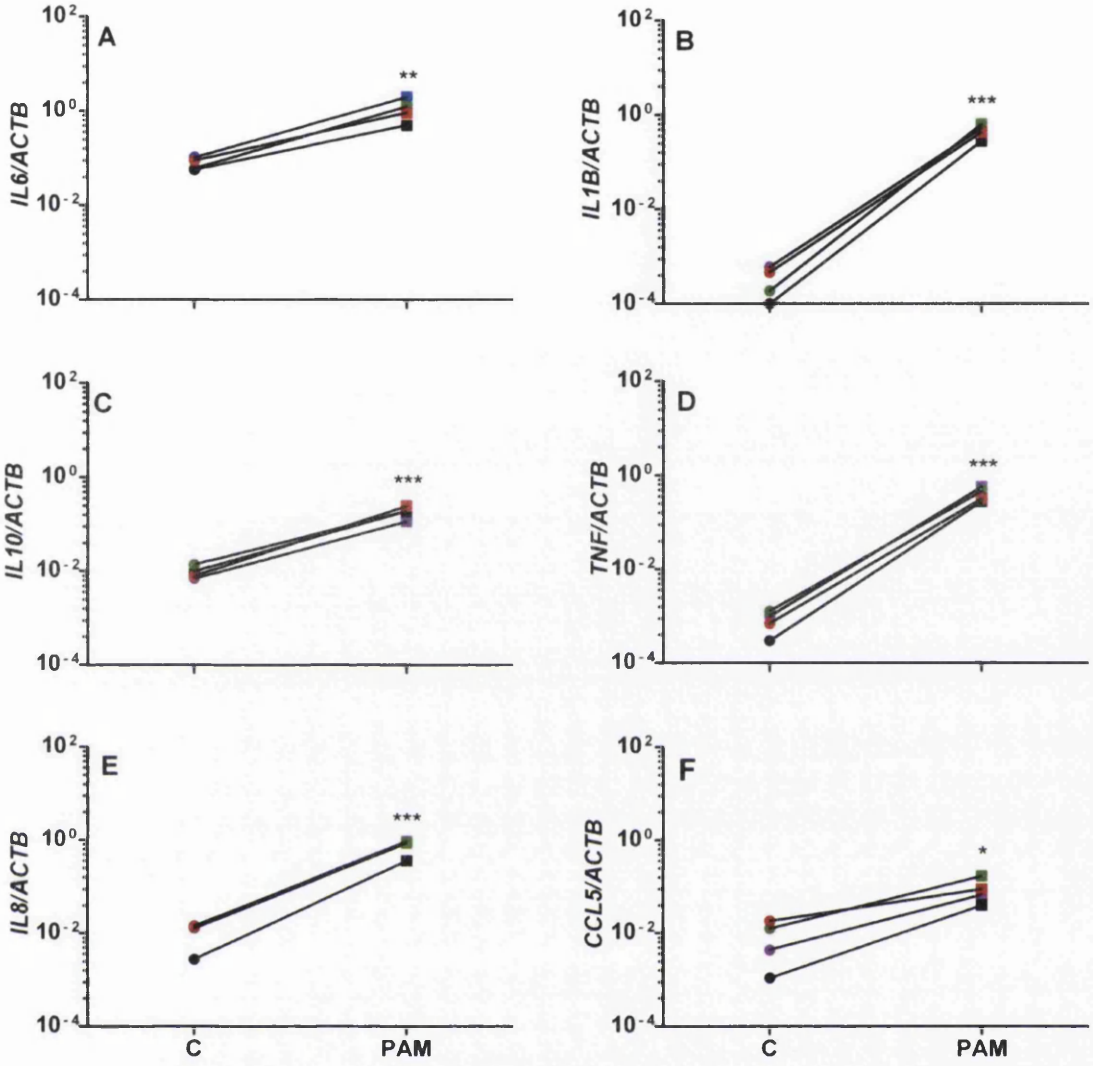


Figure 4.6 Exposure of GCs to PAM increased expression of genes associated with inflammation and innate immunity. Granulosa cells from dominant follicles were treated for 3 h with control medium ('C') or medium containing 1 μg/ml Pam3CSK4 (PAM). RNA was extracted, cDNA synthesised and the expression of *IL6* (A), *IL1B* (B), *IL10* (C), *TNF* (D), *IL8* (E) and *CCL5* (F) estimated by qPCR. Each independent experiment is presented as one line, with coloured dots representing the same experiment throughout. Mean values for PAM treatment differ from control by T-test using log-normalised data, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

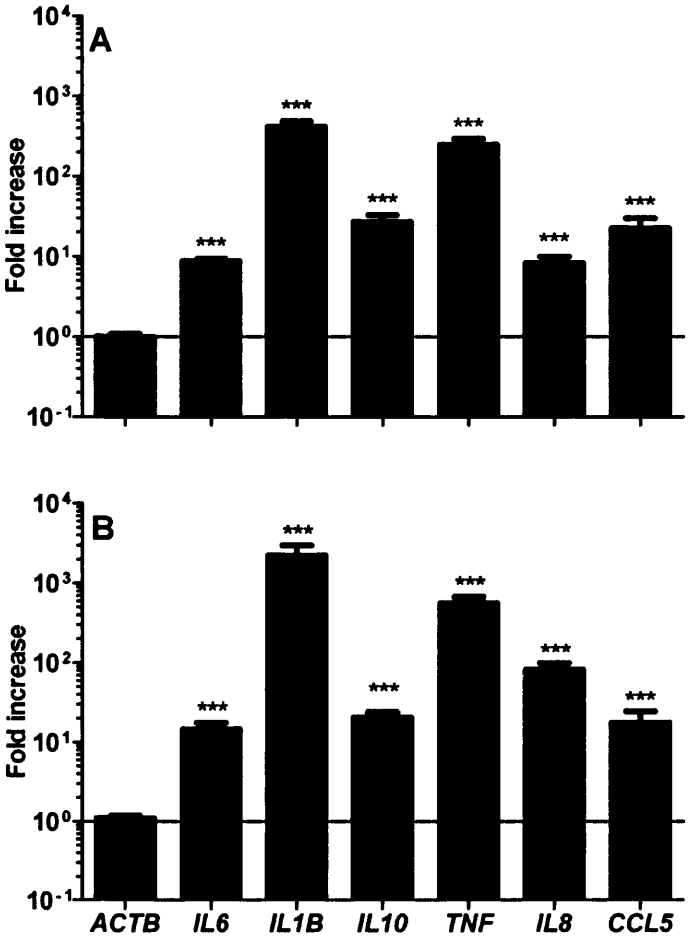


Figure 4.7 Exposure of GCs to LPS or PAM increased expression of genes associated with inflammation and innate immunity. Granulosa cells from dominant follicles were treated for 3 h with control medium or medium containing 1 μg/ml LPS (A) or 1 μg/ml PAM (B). RNA was extracted, cDNA synthesised and the expression of *ACTB, IL6, IL1B, IL10, TNF, IL8* and *CCL5* estimated by qPCR. Data are presented as mean fold increase in each gene compared to control. Data represented as mean + SEM of 3-4 independent experiments. Values differ from control by ANOVA and Dunnett's post hoc multiple comparison T-test (using $\log_{10}$ normalised data), *** $P < 0.001$

4.3.4 Treatment of GCs with PAMPs activated intracellular signalling pathways

When GCs were treated with control medium or medium containing PAMPs, 15 min LPS treatment increased phosphorylation of p38 MAPK and ERK1/2 (Fig. 4.8A-C). After 20 min PAM treatment, there was an increase in phosphorylation of p38 MAPK ($P < 0.01$, Fig. 4.9A, B) and after 10 minutes, a significant increase in phosphorylation of ERK1/2 (Fig. 4.9C), which was maintained for 15 min.

4.3.5 The response of GCs to PAMPs was attenuated using short-interfering RNA targeting TLRs

When GCs were treated with lipofectamine vehicle, scrambled siRNA, or siRNA targeting *TLR2* or *TLR4* for 24 h, there was no effect of siRNA on cell viability as evaluated by MTT test ($P > 0.5$; Fig. 4.10A). Treatment with each targeted siRNA significantly decreased expression of *TLR2* or *TLR4* mRNA ($P < 0.05$; Fig. 4.10B) and treatment with scrambled siRNA did not significantly affect expression of either target gene ($P > 0.9$). Treatment with PAM significantly increased the accumulation of IL-6, but this was attenuated when cells were pre-treated with siRNA targeting *TLR2* ($P < 0.01$; Fig. 4.10C). Treatment with LPS significantly increased the accumulation of IL-6, but this was also attenuated when cells were pre-treated with siRNA targeting *TLR4* ($P < 0.05$; Fig. 4.10D). There was no significant effect of lipofectamine vehicle, scrambled siRNA or targeted siRNA on accumulation of IL-6 by GCs treated with control medium.

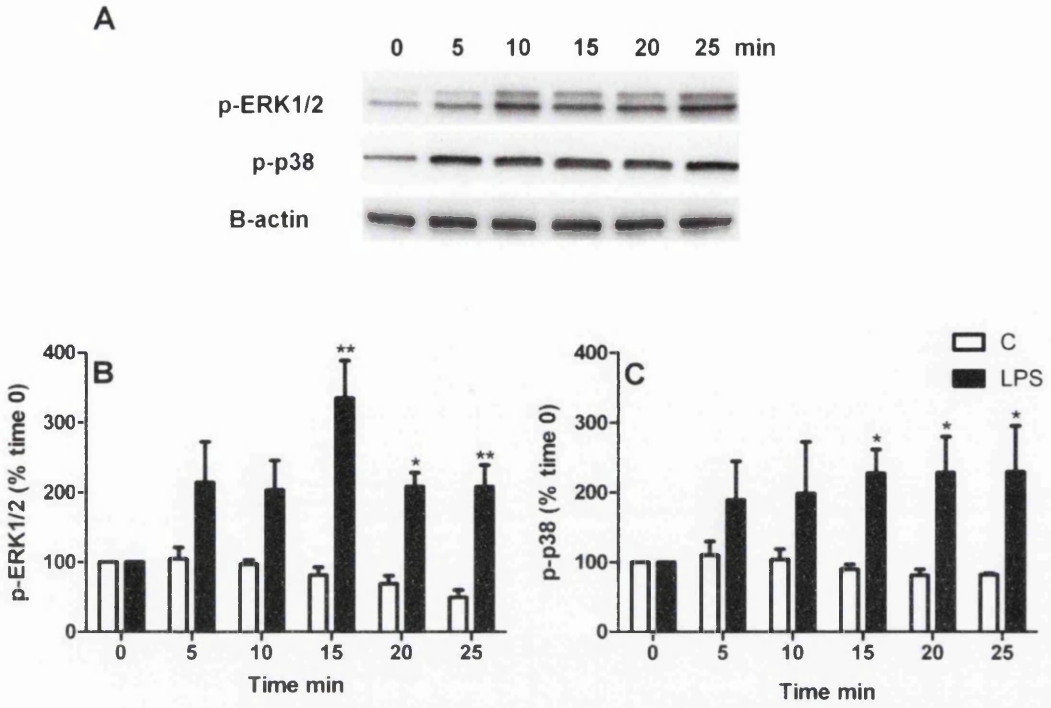


Figure 4.8 LPS induced phosphorylation of ERK1/2 and p38 MAPK in GCs. Granulosa cells from dominant follicles were treated for 5, 10, 15, 20 or 25 mins with control medium (open bars) or medium containing 1 μg/ml LPS (black bars) and phosphorylation of ERK1/2 and p38 MAPK analysed by Western blot. (A) A representative blot showing LPS treatment with bands corresponding to diphosphorylated ERK1/2, phosphorylated p38 MAPK (Thr180/182) and β-actin. Band densities were quantitated relative to β-actin and phosphorylation of ERK1/2 (B) and p38 MAPK (C) expressed as a percentage of time 0. Data presented as mean + SEM and represent 3 independent experiments. Values differ from control at each time point by t-test (log_{10} normalised data), * $P < 0.05$, ** $P < 0.01$

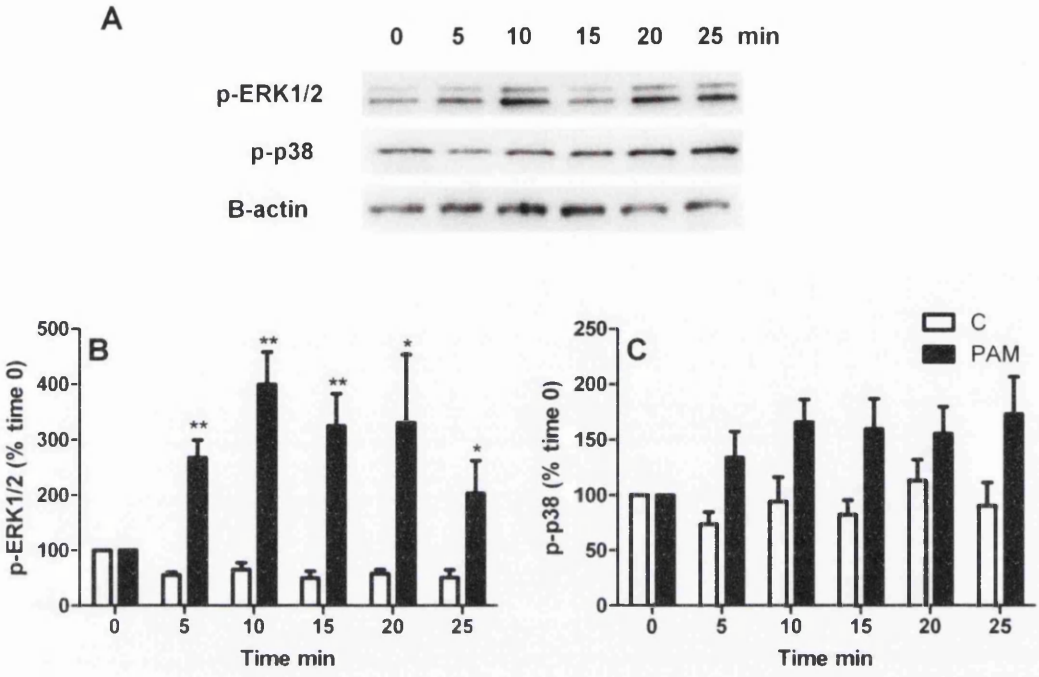


Figure 4.9 PAM induced phosphorylation of ERK1/2 and p38 MAPK in GCs. Granulosa cells from dominant follicles were treated for 5, 10, 15, 20 or 25 mins with control medium (open bars) or medium containing 1 μg/ml PAM (black bars) and phosphorylation of ERK1/2 and p38 MAPK analysed by Western blot. (A) A representative blot showing PAM treatment with bands corresponding to diphosphorylated ERK1/2, phosphorylated p38 MAPK (Thr180/182) and β-actin. Band densities were quantitated relative to β-actin and phosphorylation of ERK1/2 (B) and p38 MAPK (C) expressed as a percentage of time 0. Data presented as mean + SEM and represent 3 independent experiments. Values differ from control at each time point by t-test (log_{10} normalised data), * $P < 0.05$, ** $P < 0.01$

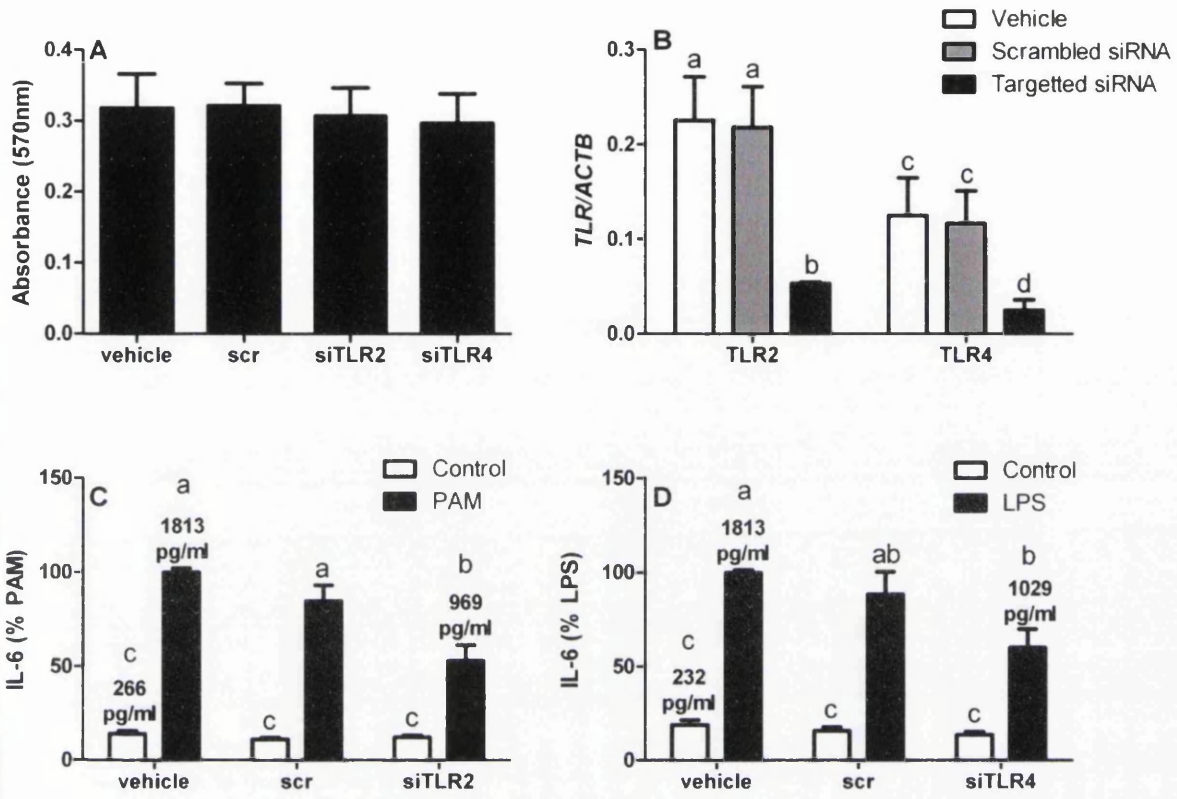


Figure 4.10 The IL-6 response of GCs to LPS or PAM was attenuated using siRNA targeting *TLR4* or *TLR2*, respectively. Granulosa cells from dominant follicles were treated for 24 h with lipofectamine (white bars), non-specific scramble-siRNA (grey bars) or specific siRNA (black bars) targeting *TLR2* or *TLR4*. The viability of the cells was assessed by MTT test (A) and expression of *TLR2* or *TLR4* estimated by qPCR (B). Data is expressed relative to *ACTB* and is presented as mean + SEM, representing 3 independent experiments. Following 24 h treatment with lipofectamine ('vehicle'), scramble-siRNA ('scr') or siRNA targeting *TLR2* ('siTLR2') or *TLR4* ('siTLR4'), GCs were treated for a further 24 h with control medium (white bars) or medium containing 1 μg/ml PAM (C) or 1 μg/ml LPS (D) and the accumulation of IL-6 measured by ELISA. Data presented as mean + SEM, expressed as percentage of PAM (C) or LPS (D) vehicle treatment and represent 4-5 independent experiments. Data that is not significantly different by ANOVA (log_{10} normalised for qPCR data; square-root normalised for ELISA data) are denoted with the same letter.

4.3.6 Inhibiting NFκB, p38 MAPK or MEK limited LPS- and PAM-stimulated IL-6 accumulation by GCs

To explore which intracellular signalling pathways are important for the inflammatory response to LPS and PAM, GCs were pre-treated with chemical inhibitors that target NFκB or specific MAPK. There was a reduction in the accumulation of IL-6 in culture supernatants in response to LPS, without a significant impact on cell survival, when cells were pre-treated with 0.04 – 4 μM NFκB inhibitor (Fig. 4.11A), 5 μM JNK inhibitor (Fig. 4.11C), 1 – 10 μM MEK inhibitor (Fig. 4.11D) or 0.001 – 10 μM p38 MAPK inhibitor (Fig. 4.11E). Similarly, there was a reduction in the accumulation of IL-6 in culture supernatants in response to PAM, without a significant impact on cell survival, when cells were pre-treated with 0.4 μM NFκB inhibitor (Fig. 4.12A), 50 μM JNK inhibitor (Fig. 4.12C), 1 – 10 μM MEK inhibitor (Fig. 4.12D) or 1 – 10 μM p38 MAPK inhibitor (Fig. 4.12E). There was no reduction in IL-6 accumulation without a corresponding effect on cell health when a PKC inhibitor was used (Fig. 4.11B, 4.12B).

Toward a therapeutic approach, pairs of inhibitors were also tested using the lowest concentration of each inhibitor in order to minimise the impact on granulosa cell health. The combination of inhibitors targeting p38 MAPK (0.001 μM) and MEK (0.0001 μM) limited the accumulation of IL-6 in response to LPS (Fig. 4.11F). However, there was no significant effect of the pairs of inhibitors on the response to PAM (Fig. 4.12F).

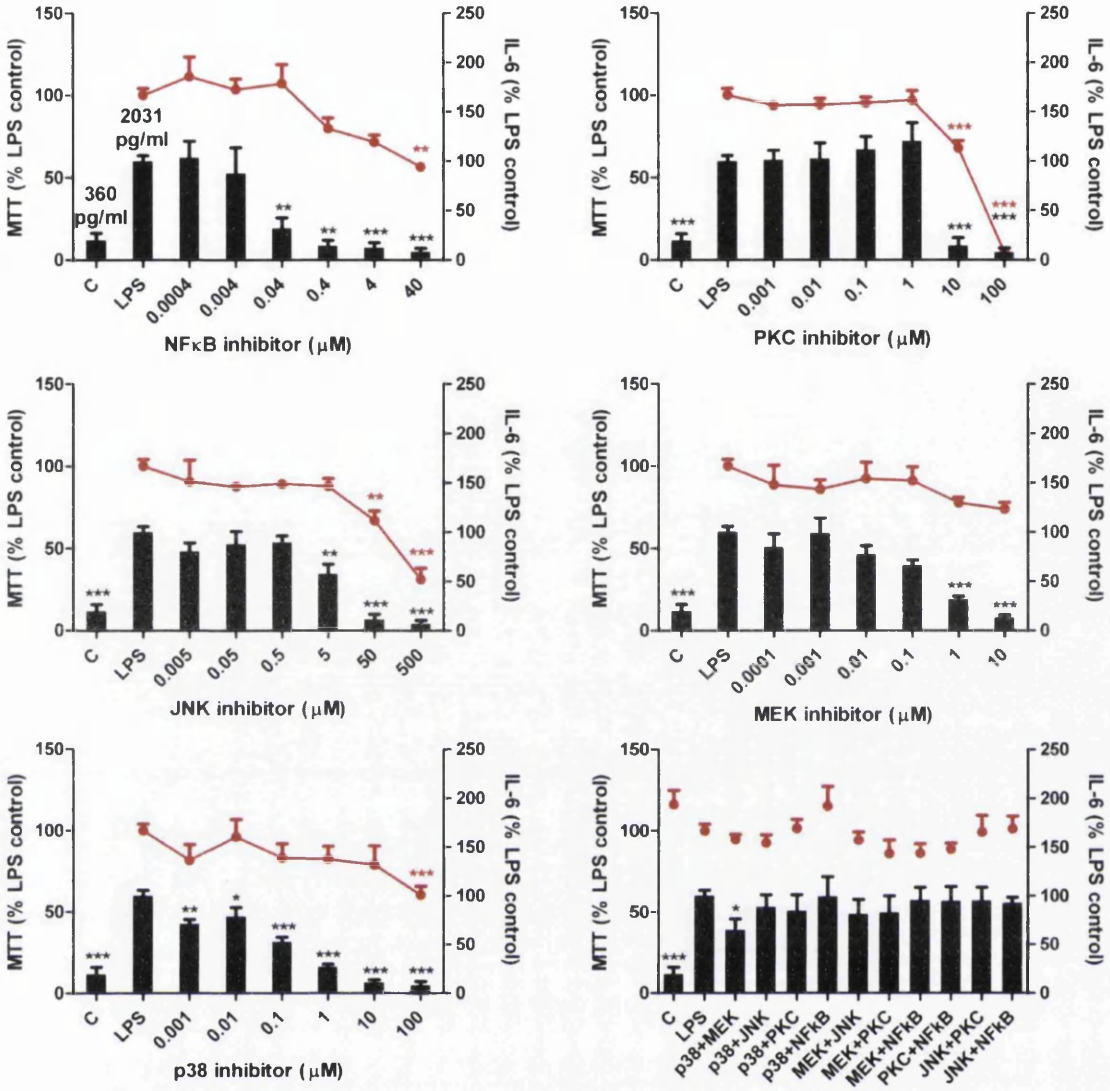


Figure 4.11 Inhibiting NFκB, MEK or p38 MAPK limited LPS-induced IL-6 accumulation by GCs. Granulosa cells were treated for 30 min with 10-fold increasing concentrations of biochemical inhibitors targeting NFκB (A; 0.0004 to 40 μM), PKC (B; 0.001 to 100 μM), JNK (C; 0.005 to 500 μM), MEK (D; 0.0001 to 10 μM), p38 MAPK (E; 0.001 to 100 μM) or low dose combination treatments using pairs of inhibitors (0.0004 μM NFκB inhibitor, 0.001 μM PKC inhibitor, 0.005 μM JNK inhibitor, 0.0001 μM MEK inhibitor and 0.001 μM p38 MAPK inhibitor); followed by addition of 1 μg/ml LPS (final concentration) to each well for 24 h. Supernatants were collected and the accumulation of IL-6 measured by ELISA (black bars). Cell viability was assessed by MTT assay (red line). Data presented as mean + SEM from 3-4 independent experiments and are expressed as a percentage of LPS treatment. Values differ from LPS treatment by ANOVA and Dunnett's pairwise multiple comparison t test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

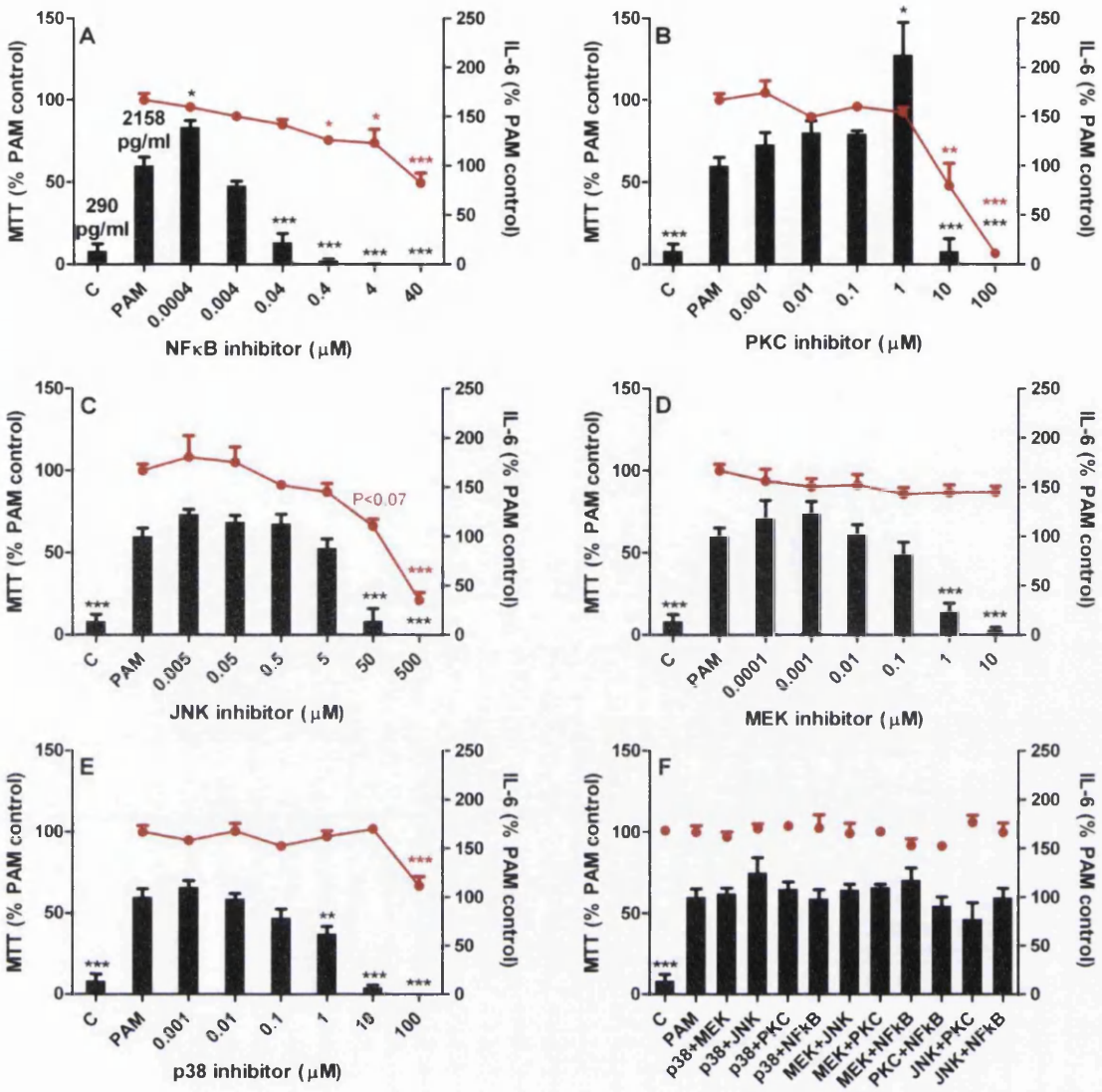


Figure 4.12 Inhibiting NFκB, MEK or p38 MAPK limited PAM-induced IL-6 accumulation by GCs. Granulosa cells were treated for 30 min with 10-fold increasing concentrations of biochemical inhibitors targeting NFκB (A; 0.0004 to 40 μM), PKC (B; 0.001 to 100 μM), JNK (C; 0.005 to 500 μM), MEK (D; 0.0001 to 10 μM), p38 MAPK (E; 0.001 to 100 μM) or low dose combination treatments using pairs of inhibitors (0.0004 μM NFκB inhibitor, 0.001 μM PKC inhibitor, 0.005 μM JNK inhibitor, 0.0001 μM MEK inhibitor and 0.001 μM p38 MAPK inhibitor); followed by addition of 1 μg/ml PAM (final concentration) to each well for 24 h. Supernatants were collected and the accumulation of IL-6 measured by ELISA (black bars). Cell viability was assessed by MTT assay (red line). Data presented as mean + SEM from 3-4 independent experiments and are expressed as a percentage of PAM treatment. Values differ from PAM treatment by ANOVA and Dunnett's pairwise multiple comparison t test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.3.7 Exposure of GCs to LPS or PAM impacted endocrine function

To investigate the effect of PAMPs on endocrine function, GCs were collected after 3 h treatment with LPS or PAM to examine mRNA expression. Treatment of cells with LPS reduced the expression of mRNA encoding *EGFR* and *CYP19A1* but not *FSHR* or *LHCGR* (Fig. 4.13, 4.14). Treatment of GCs with PAM decreased the mRNA expression of *LHCGR* and *CYP19A1* and tended towards decreasing expression of *EGFR* (0.86 ± 0.06 fold change; $P < 0.1$) but did not affect *FSHR* expression (Fig. 4.13, 4.14).

To investigate the effect of PAMPs on hormone production, GCs were treated with FSH and androstenedione. Cell supernatants accumulated oestradiol and progesterone in a time-dependent manner with significant increases in oestradiol from 2 h and significant increases in progesterone from 24 h. Exposure to LPS or PAM for 24 h reduced the accumulation of oestradiol (Fig.4.15B-D) and progesterone (Fig.4.16B-D) in culture supernatants.

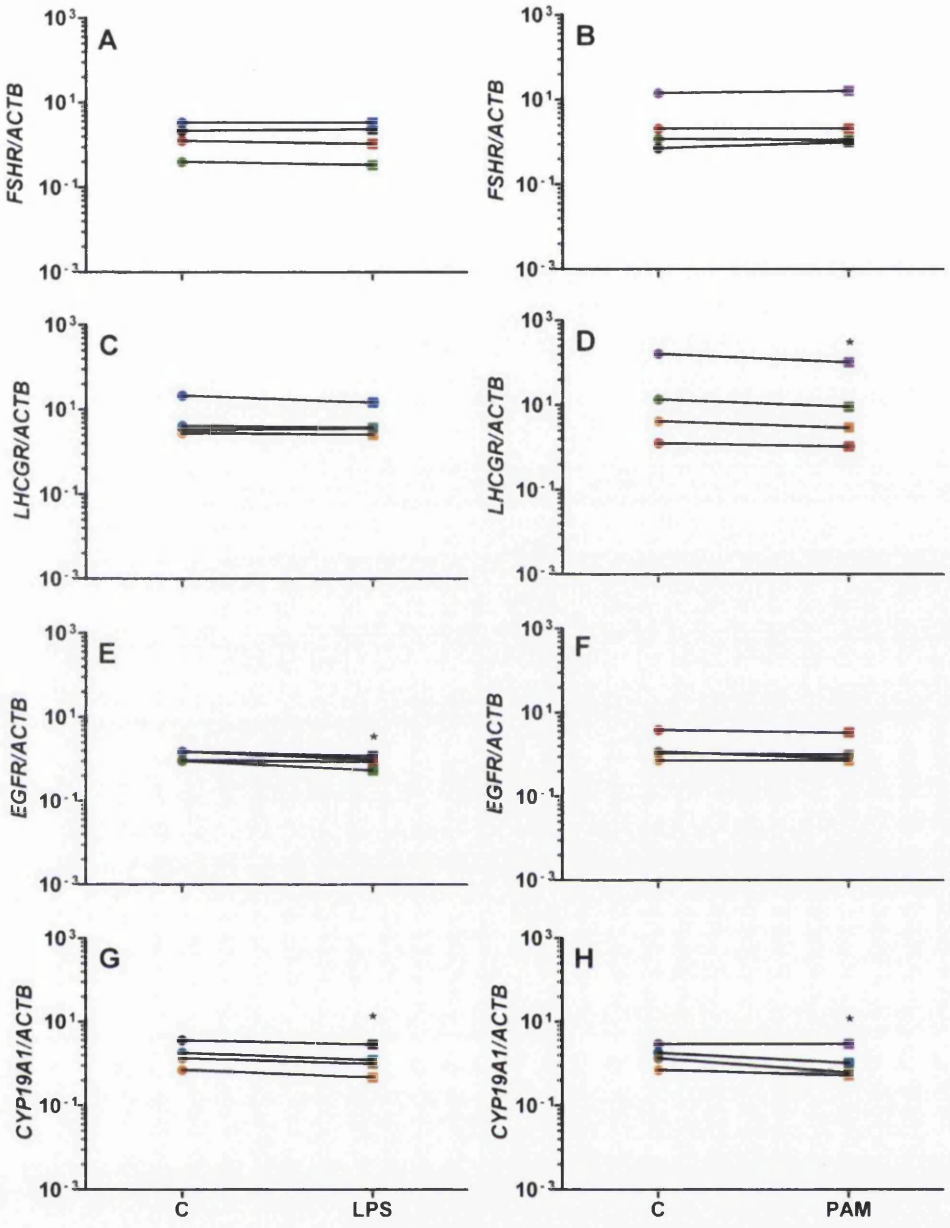


Figure 4.13 Exposure of GCs to LPS or PAM decreased expression of genes associated with reproductive function. Granulosa cells from dominant follicles were treated for 3 h with control medium ('C') or medium containing 1 μg/ml LPS (A, C, E, G) or PAM (B, D, F, H). RNA was extracted, cDNA synthesised and the expression of *FSHR* (A, B), *LHCGR* (C, D), *EGFR* (E, F) and *CYP19A1* (G, H) estimated by qPCR. Each independent experiment is presented as one line, with coloured dots representing the same experiment throughout. Mean values for LPS or PAM treatment differ from control by T-test using log-normalised data, * $P < 0.05$.

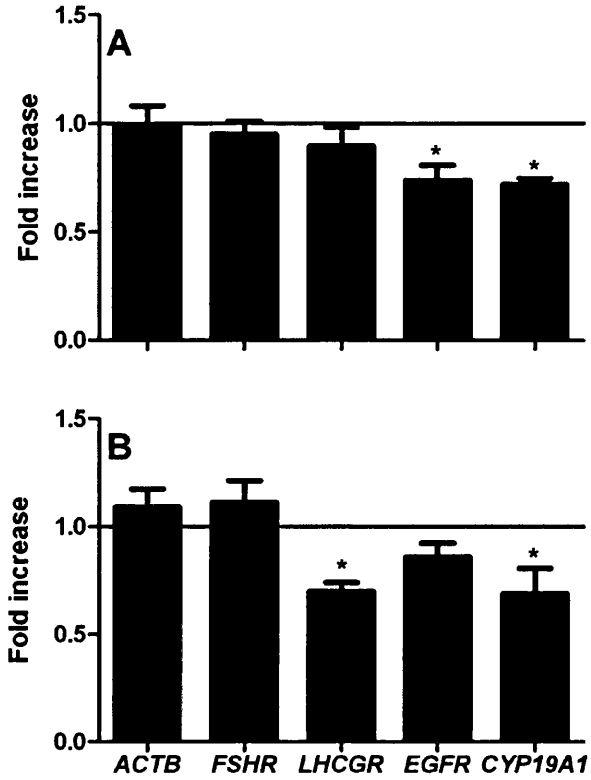


Figure 4.14 Exposure of GCs to LPS or PAM decreased expression of genes associated with reproductive function. Granulosa cells from dominant follicles were treated for 3 h with control medium or medium containing 1 μg/ml LPS (A) or 1 μg/ml PAM (B). RNA was extracted, cDNA synthesised and the expression of *ACTB, FSHR, LHCGR, EGFR* and *CYP19A1* estimated by qPCR. Data are presented as mean fold increase in each gene compared to control. Data represented as mean + SEM of 3-4 independent experiments. Values differ from control by ANOVA and Dunnett's post hoc multiple comparison T-test (using $\log_{10}$ normalised data), * $P < 0.05$.

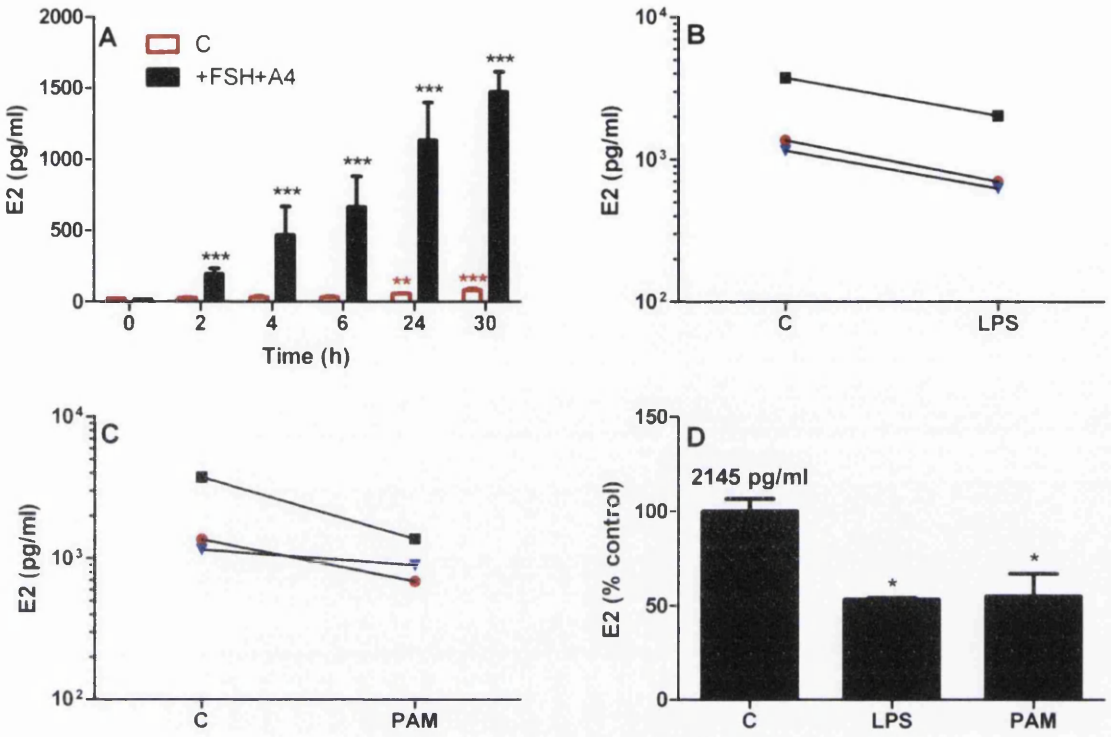


Figure 4.15 Exposure of GCs to LPS or PAM decreased production of oestradiol. (A) Granulosa cells from dominant follicles were treated for 0, 2, 4, 6, 24 or 30 h with control medium (red open bars) or medium containing 1 ng/ml FSH and 10^{-7} M androstenedione (black bars). Supernatants were collected and the accumulation of oestradiol measured by ELISA. Data presented as mean + SEM and differ from time 0 within treatment group by ANOVA and Dunnett's post hoc multiple comparison T-test (log_{10} normalised data), ** $P < 0.01$, *** $P < 0.001$. Granulosa cells were then treated for 24 h with control medium containing 1 ng/ml FSH and 10^{-7} M androstenedione ('C') or control medium also containing 1 µg/ml LPS (B) or 1 µg/ml PAM (C). Supernatants were collected and the accumulation of oestradiol measured by ELISA. Each independent experiment is presented as one line, with coloured dots representing the same experiment throughout. (D) Data presented as mean + SEM, expressed as a percentage of control oestradiol accumulation and represent 3 independent experiments; values differ from control by Mann-Whitney U test; * $P < 0.05$.

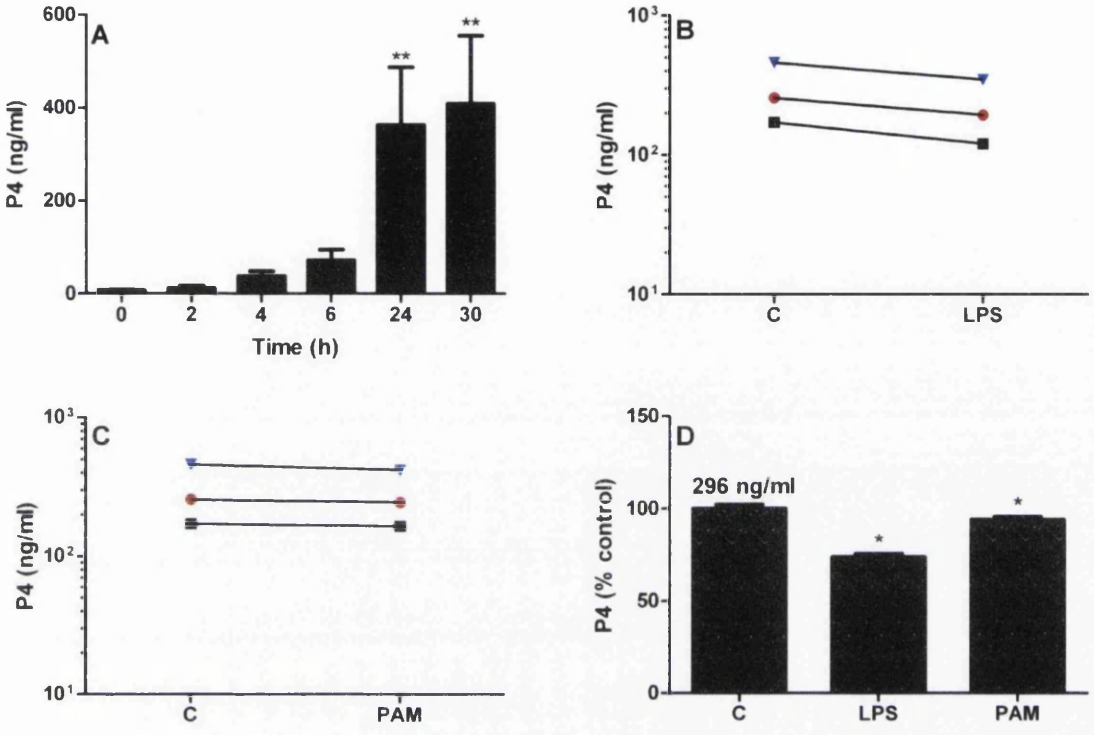


Figure 4.16 Exposure of GCs to LPS or PAM decreased production of progesterone. (A) Granulosa cells from dominant follicles were treated for 0, 2, 4, 6, 24 or 30 h with medium containing 1 ng/ml FSH and 10^{-7} M androstenedione. Supernatants were collected and the accumulation of progesterone measured by ELISA. Data presented as mean + SEM and differ from time 0 by ANOVA and Dunnett's post hoc multiple comparison T-test (square root normalised data), ** $P < 0.01$. Granulosa cells were then treated for 24 h with control medium containing 1 ng/ml FSH and 10^{-7} M androstenedione ('C') or control medium also containing 1 μg/ml LPS (B) or 1 μg/ml PAM (C). Supernatants were collected and the accumulation of progesterone measured by ELISA. Each independent experiment is presented as one line, with coloured dots representing the same experiment throughout. (D) Data presented as mean + SEM, expressed as a percentage of control progesterone accumulation and represent 3 independent experiments; values differ from control by Mann-Whitney U test; * $P < 0.05$.

4.3.8 EGF increased accumulation of IL-6 by GCs

Previously, it was shown that treatment with PAMPs impacts the hormonal output of GCs (Fig. 4.16). It was also of interest whether the endocrine environment affected the inflammatory output of the GCs from dominant follicles. Thus, GCs were treated for 24 h with control medium (Fig. 4.17) or medium containing LPS (Fig. 4.18) or PAM (Fig. 4.19) in combination with 100 ng/ml FSH, 4 ng/ml LH, 500 ng/ml E2, 250 ng/ml P4 or 10 ng/ml EGF. Treatment with LH or EGF significantly increased accumulation of IL-6 by GCs treated in control medium (Fig. 4.17A). Treatment with EGF also significantly increased accumulation of IL-6 by GCs treated in LPS or PAM medium (Fig. 4.18A, 4.19A). Oestradiol treatment significantly decreased IL-6 accumulation by GCs stimulated by LPS or PAM but had no effect on GCs treated in control medium ($P > 0.6$). FSH, LH and P4 had no significant effect on IL-6 accumulation by GCs. There was no effect of endocrine treatment on accumulation of IL-1β or IL-8, or on cell viability measured by MTT test ($P > 0.9$).

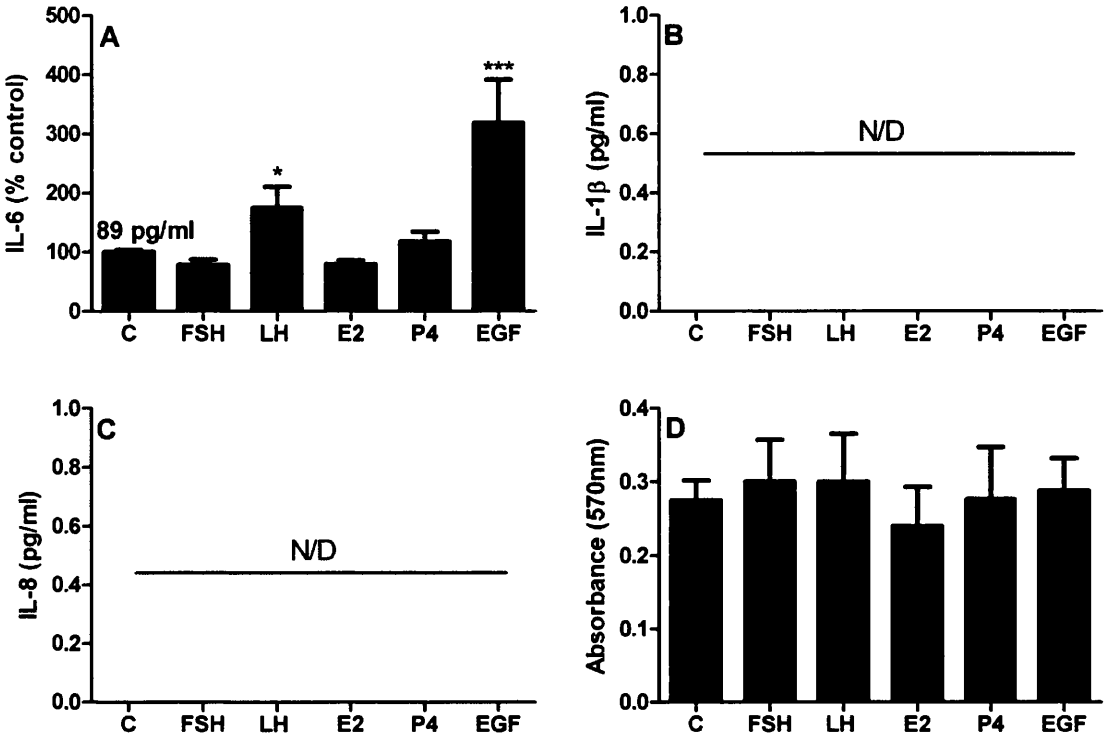


Figure 4.17 LH or EGF increased accumulation of IL-6 by GCs from dominant follicles. Granulosa cells from dominant follicles were treated for 24 h with control medium ('C') or medium containing 100 ng/ml FSH, 4 ng/ml LH, 500 ng/ml E2, 250 ng/ml P4 or 10 ng/ml EGF. Supernatants were collected and accumulation of IL-6 (A), IL-1β (B) and IL-8 (C) measured by ELISA. Data are presented as mean + SEM and represent at least 4 independent experiments. For IL-6 values, data are expressed as a percentage of control. Values differ from control by ANOVA and Dunnett's pairwise multiple comparison t-test (log_{10} normalised data), * $P < 0.05$, *** $P < 0.001$. (D) Cell viability was assessed by MTT test. Data presented as mean + SEM from 3 independent experiments. N/D = below limits of detection.

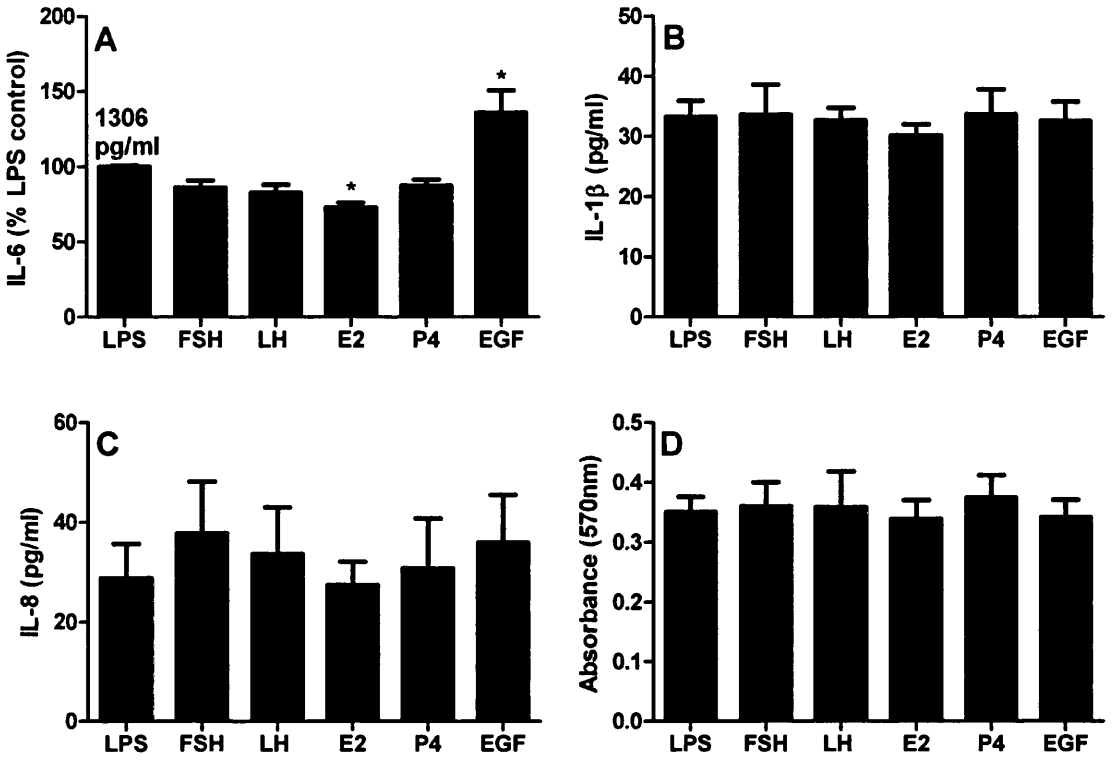


Figure 4.18 EGF increased accumulation of IL-6 by GCs from dominant follicles treated with LPS; oestradiol decreased IL-6 accumulation. Granulosa cells from dominant follicles were treated for 24 h with medium containing 1 μg/ml LPS or medium containing 1 μg/ml LPS plus 100 ng/ml FSH, 4 ng/ml LH, 500 ng/ml E2, 250 ng/ml P4 or 10 ng/ml EGF. Supernatants were collected and accumulation of IL-6 (A), IL-1β (B) and IL-8 (C) measured by ELISA. Data are presented as mean + SEM and represent at least 4 independent experiments. For IL-6 values, data are expressed as a percentage of LPS treatment alone. Values differ from control by ANOVA and Dunnett's pairwise multiple comparison t-test (log_{10} normalised data), * $P < 0.05$. (D) Cell viability was assessed by MTT test. Data presented as mean + SEM from 3 independent experiments.

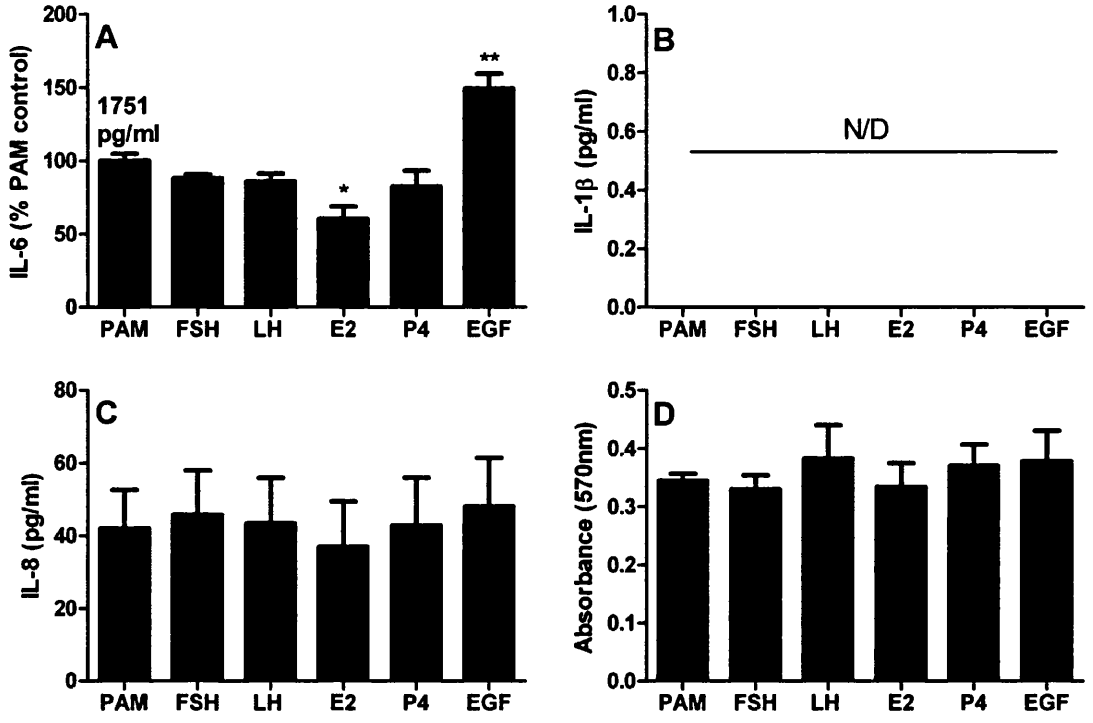


Figure 4.19 EGF increased accumulation of IL-6 by GCs from dominant follicles treated with PAM; oestradiol decreased IL-6 accumulation. Granulosa cells from dominant follicles were treated for 24 h with medium containing 1 μg/ml PAM or medium containing 1 μg/ml PAM plus 100 ng/ml FSH, 4 ng/ml LH, 500 ng/ml E2, 250 ng/ml P4 or 10 ng/ml EGF. Supernatants were collected and accumulation of IL-6 (A), IL-1β (B) and IL-8 (C) measured by ELISA. Data are presented as mean + SEM and represent at least 4 independent experiments. For IL-6 values, data are expressed as a percentage of PAM treatment alone. Values differ from control by ANOVA and Dunnett's pairwise multiple comparison t-test (log_{10} normalised data), * $P < 0.05$, ** $P < 0.01$. (D) Cell viability was assessed by MTT test. Data presented as mean + SEM from 3 independent experiments. N/D = below limits of detection.

4.4 Discussion

Bacterial infections of the uterus or mammary gland perturb ovarian dominant follicle growth and endocrine function (Sheldon et al., 2002, Lavon et al., 2011a, Lavon et al., 2011b). Whilst LPS has been found in the follicular fluid from dominant follicles of animals with uterine disease (Herath et al., 2007), healthy ovarian follicles are devoid of immune cells (Spanel-Borowski et al., 1997, Bromfield and Sheldon, 2011). So, the present study considered whether the GCs inside dominant follicles may have roles in innate immunity. Primary GCs collected from dominant follicles expressed mRNA for all ten TLRs, and produced IL-1β, IL-6 and IL-8 in response to PAM and LPS via the TLR2 and TLR4 pathways, respectively. However, the cells did not accumulate cytokines in response to the TLR5 ligand, flagellin. The GCs had rapid responses to PAM or LPS, with increased abundance of *IL6, IL1B, IL10, TNF, IL8* and *CCL5* mRNA, and increased phosphorylation of p38 MAPK and ERK1/2. Furthermore, treating GCs with inhibitors targeting MAPK or NFκB reduced the cellular response to these PAMPs. Treatment with LPS or PAM also reduced the accumulation of oestradiol and progesterone, and the expression of genes important for granulosa cell endocrine function. The inflammatory responses to PAMPs via TLR2 and TLR4 pathways by GCs provides a molecular explanation of how bacterial infections distant to the ovary may perturb ovarian dominant follicle function.

In the present study, FACS and PCR analysis were used to confirm that the preparations of GCs isolated from dominant follicles were free from immune cell contamination, as determined by lack of *CD45* and *MHCII* expression, because such contaminations might have confounded the investigations (chapter 2). The bovine GCs from dominant follicles expressed mRNA encoding all ten TLRs and the gene expression was maintained during cell culture. Although levels of TLR gene expression may have little bearing on immune function, the high expression of *TLR3* was notable. This is interesting because viruses such as bovine viral diarrhoea virus infect the ovary of cattle and perturb dominant follicle growth (Grooms et al., 1998). The high expression of *TLR1* and *TLR6* was also noticeable, as these receptors dimerise with TLR2 to recognise bacterial lipoproteins (Kawai and Akira, 2011). However, the granulosa cell expression of *TLR2* and *TLR4* was of immediate

relevance as these receptors are required to detect the purified PAMPs used in the present study. Pre-treatment of GCs with LPS or PAM increased both expression of *TLR2* and, in contrast to GCs from emerged follicles, the cellular response to further PAM treatment, suggesting that PAMPs have the ability to prime GCs from dominant follicles to respond to other TLR ligands. Supporting this hypothesis that LPS primes cells to respond to TLR2 ligands, LPS pre-treatment also increases IL-6 accumulation by murine bone-marrow derived macrophages subsequently treated with the TLR2/TLR6 agonist FSL-1 (Schroder et al., 2012), and pre-treatment of dendritic cells with LPS increases IL-6 production by cells subsequently challenged with PAM (Geisel et al., 2007).

The cellular response of GCs isolated from dominant follicles to PAMPs was similar to that of GCs isolated from emerged follicles. Supernatants of GCs from dominant follicles treated with LPS or PAM for 24 h accumulated the cytokines IL-1β and IL-6, and the chemokine IL-8. In addition to protein accumulation, the GCs mounted acute responses after 3h treatment with LPS and PAM, characterised by increased expression of gene transcripts for the cytokines *IL6, IL1B, IL10* and *TNF* and the chemokines *IL8* and *CCL5*. The changes in inflammatory mediators found in the present study are typical of those associated with an innate immune response to LPS or PAM in immune cells such as macrophages and dendritic cells (Akira et al., 2006, Takeuchi and Akira, 2010, Moresco et al., 2011). Interestingly, many of the inflammatory mediators associated with innate immunity also have physiological functions in the ovary. For example, IL-6 induces murine cumulus-oocyte-complex expansion (Liu et al., 2009) and IL-1β decreases progesterone production by FSH-treated murine GCs (Santana et al., 1996). Chemokines also play a role, with IL-8 involved in follicle development, ovulation and corpus luteum formation (Goto et al., 2002). The absence of TNFα protein in the present study might be considered surprising for some tissues and immune cells; however, changes in protein are less often detected than changes in *TNF* gene expression in cells of the bovine reproductive tract (Swangchan-Uthai et al., 2012). The requirement for TLR2 and TLR4 in bovine GCs to produce IL-6 in response to PAM and LPS was confirmed using siRNA targeting *TLR4* or *TLR2*, respectively. Although TLR2 has not been investigated, bovine GCs from emerged follicles before dominance similarly required TLR4 to respond to LPS (Bromfield and Sheldon, 2011).

In immune cells, binding of bacterial PAMPs to TLRs activates NFκB and MAPK pathways, leading to production of inflammatory mediators (Akira et al., 2006). In the present study, treatment of GCs from dominant follicles with LPS or PAM increased the abundance of phosphorylated ERK1/2 and p38 MAPK within 20 min. These observations are similar to those in bovine GCs collected from follicles before dominance and treated with LPS (Bromfield and Sheldon, 2011). However, to extend these observations, the present study also explored the effect of chemical inhibitors that target NFκB, JNK, MEK or p38 MAPK on the accumulation of LPS or PAM-stimulated IL-6. Indeed, concentrations of the inhibitors targeting NFκB, JNK, MEK or p38 MAPK were identified that reduced the TLR4- or TLR2-mediated inflammatory response, without significantly reducing granulosa cell survival. An approach to limiting the risk of chemical cytotoxicity when designing therapeutics is to use combinations of inhibitors at minimal concentrations (Small et al., 2011). In the present study, this approach identified a combination of inhibitors targeting p38 MAPK and MEK that limited the accumulation of LPS-stimulated IL-6, with no significant effect on granulosa cell viability. Although beyond the scope of the present work, to move toward therapeutics it would also be important to explore whether these chemical inhibitors modulated granulosa cell steroidogenesis. Previously, it was shown that GCs from bovine dominant follicles have greater abundance of phosphorylated ERK1/2 than GCs from smaller follicles (Ryan et al., 2007). As ERK1/2 may be involved in follicular development, and treatment of GCs with PAMPs increased phosphorylation of ERK1/2, it is possible that PAMP treatment may increase the rate of follicular development, although not from primordial follicles, whose rate of follicular atresia increases (Bromfield and Sheldon, 2013).

Cows with uterine disease or mastitis have lower peripheral oestradiol concentrations and delayed ovulation *in vivo* (Sheldon et al., 2009, Lavon et al., 2011b). The accumulation of oestradiol and progesterone is decreased when GCs from dominant follicles are treated with 1 μg/ml LPS (Herath et al., 2007). Similarly, in the present study, there was a ~50% decrease in oestradiol accumulation and qPCR was used to measure a ~30% decrease in *CYP19A1* expression by GCs from dominant follicles after treatment with LPS or PAM. It is likely that these changes

would have a detrimental impact on ovarian follicle growth and the likelihood of ovulation.

Progesterone accumulation was also impacted with LPS treatment, an effect also observed after *in vivo* infusion of LPS into bovine mammary gland (Lavon et al., 2011b). This lower progesterone concentration further decreases the chance of successful conception, as the endometrium will be affected. Progesterone tends to have an anti-inflammatory effect; for example, macrophages treated with progesterone produce significantly less IL-6 when stimulated with LPS compared to macrophages treated with either oestradiol or control medium (Su et al., 2009). Thus, lower progesterone would favour a pro-inflammatory response. This could be useful to the animal to help clear the infection. However, inappropriate inflammation increases tissue damage and decreases the likelihood of conception. In contrast to the effect of LPS on progesterone accumulation, the statistically significant decrease in progesterone accumulation caused by PAM is less likely to be of biological significance.

In the present study, LPS also decreased expression of *EGFR* and treatment with PAM decreased expression of *LHCGR*, which might further perturb ovarian function. Links between LPS and the EGF system have been observed previously: LPS increases expression of EGFR ligands amphiregulin and epiregulin (Brandl et al., 2010). Activation of EGFR leads to endocytosis of the receptor, followed by either degradation or recycling (Madshus and Stang, 2009). Thus, a negative feedback loop may down-regulate *EGFR* gene expression following receptor internalisation. Further links between the EGF system and infection were demonstrated in this study, as treatment of GCs with EGF increased IL-6 accumulation and increased LPS- or PAM- stimulated IL-6 accumulation. This pro-inflammatory effect of EGF is consistent with previous studies using mesenchymal stem cells (Kerpedjieva et al., 2012) and ovarian cancer cells (Colomiere et al., 2008). Treatment of murine COCs with the EGF-like factor amphiregulin increases expression of *Myd88* and other TLR-associated genes (Shimada et al., 2006), which in turn could increase the response of COCs to PAMPs. However, in the same study, amphiregulin did not increase the expression of *Myd88* or *TLR4* in murine GCs (Shimada et al., 2006). It would be interesting in future work to examine the effect of EGF, hormones and gonadotrophins on *TLR*

expression in bovine GCs and the effect of PAMPs on production of amphiregulin and epiregulin.

Oestradiol decreased PAMP stimulated IL-6 accumulation, but not IL-6 accumulation by GCs in control medium. These results are not surprising, as it is unlikely that basal IL-6 accumulation would be reduced and oestradiol has many anti-inflammatory actions on a variety of cell types including bone cells (Kurebayashi et al., 1997) and in the brain (Vegeto et al., 2002). Supporting the findings of the present study, treatment of murine macrophages with oestradiol down-regulates LPS-induced *Il6* and *Tnf* expression (Deshpande et al., 1997) and dampens the LPS-stimulated production of the chemokine MCP-1 by murine macrophages (Frazier-Jessen and Kovacs, 1995). A potential mechanism for this dampening is the suppression of NFκB activation, perhaps through influencing translocation of the p65 subunit to the nucleus (Ghisletti et al., 2005) or by increasing expression of a micro-RNA inhibitor of NFκB (Murphy et al., 2010).

Interestingly, LH increased IL-6 accumulation when GCs were not treated with a PAMP, but LH did not increase LPS- or PAM- stimulated IL-6 accumulation. Published results supporting this finding that LH increases IL-6 accumulation include a study which examined the response of ovarian epithelial and cancer cell-lines to treatment with gonadotrophins and noted increased *IL6* mRNA and protein (Syed et al., 2002). A more recent study mimicked the LH surge using cAMP and found this increased *Il6* mRNA expression in murine GCs (Mack et al., 2012). Signalling cascades induced by LH lead to increased production of EGFR ligands (Liu et al., 2008). Thus, the increased production of IL-6 by LH could be purely because an EGF-like factor is produced. Potentially, the lack of an increased IL-6 response when GCs are treated with LH in addition to a PAMP compared to PAMP treatment alone could be through interference of the PAMP in the cAMP and PKC LH signalling pathway, thus preventing the EGFR ligand being produced (Freimann et al., 2004).

In conclusion, bovine GCs from dominant follicles expressed functional TLR4 and TLR2, but not TLR5. Granulosa cells produced cellular responses to LPS and PAM, with increased expression and accumulation of inflammatory cytokines and chemokines, activation of MAPK pathways, and perturbation of steroidogenesis. There is potential for the development of therapeutic approaches as the inflammatory responses to bacterial PAMPs were blunted using biochemical inhibitors targeting NFκB and MAPK. The present study supports the hypothesis that GCs from dominant follicles play a role in innate immunity in the ovary.

Chapter 5

The impact of bacterial infections on the oocyte and embryo

5.1 Introduction

In the previous chapters, the effects of PAMPs on GCs from both emerged and dominant follicles were investigated. Lipopolysaccharide collects in the follicular fluid of cows with uterine disease (Herath et al., 2007) and GCs respond to this LPS, producing IL-6 (chapters 3 and 4). This means that the oocyte is bathed in a cocktail of LPS (it is not currently known if other PAMPs collect in follicular fluid) and inflammatory mediators. Although the effect of LPS and IL-6 on murine oocytes has been investigated (Shimada et al., 2006, Liu et al., 2009), less is known about the effect of PAMPs on the bovine oocyte and developing embryo. Thus, it was of interest whether PAMPs have a direct effect on the cumulus-oocyte complex (COC), or only an effect through perturbed mural granulosa cytokine production.

Ovulation is the process by which an oocyte is released by the dominant follicle in response to a surge in LH, released from the pituitary gland. The ovarian surface epithelium ruptures, releasing the oocyte surrounded by cumulus cells. The remaining mural GCs of the follicle become luteinised and the remainder of the primary follicle forms a highly vascularised corpus luteum, which produces progesterone. The LH surge induces expression of EGF-like factors by GCs (Park et al., 2004), which propagate the LH signal across the follicle to the COC, leading to resumption of meiosis in the oocyte and formation of a hyaluronan-rich extracellular matrix, necessary for cumulus expansion (Fulop et al., 2003). The LH surge also stimulates plasminogen activator, producing bioactive plasmin (LeMaire, 1989), which then activates collagenase (Woessner Jr et al., 1989), digesting the follicular tissue to form a hole in the follicle wall, through which the COC can ovulate.

Ovulation is similar to an inflammatory response on a number of levels. At the gross anatomical level, the site of ovulation appears swollen and red and the process damages the ovarian surface epithelium and vasculature, triggering the coagulation cascade and remodelling tissue in a similar way to wound repair. At the biochemical level, a number of inflammatory mediators, including pro-inflammatory cytokines and prostaglandins, are involved in both ovulation and more classical inflammation. The cytokine IL-1 induces prostaglandin synthesis (Gerard et al., 2004) and PGE increases protein levels of plasminogen activator in GCs, which acts proteolytically to aid ovulation (Markosyan and Duffy, 2009). Supporting the theory

that prostaglandins commonly associated with inflammation play a role in follicle rupture, inhibitors of prostaglandin synthesis block ovulation (Kaur et al., 1986). Prostaglandin E_2 (PGE_2) also acts in combination with EGF factors to trigger cumulus expansion. The chemokine IL-8 is a potent attractant of neutrophils and may be involved in follicle rupture and angiogenesis during ovulation (Runesson et al., 2000).

A number of studies have identified molecular similarities between ovulation and immunity or inflammation. In GCs, LH increases production of EGF factors through p38 MAPK and PKA (Yamashita et al., 2009). These then act through the EGFR and ERK1/2 pathways to increase expression of mRNA encoding *PTGS2* (also known as *COX2*), which converts arachidonic acid to prostaglandins (Yamashita et al., 2009, Sayasith et al., 2013). These pathways (p38 MAPK and ERK1/2) were identified in chapters 3 and 4 as key signals in the response of GCs to LPS and PAM. Luteinising hormone also induces genes involved with production of hyaluronan rich matrix, such as hyaluronan synthase 2 (*HAS2*) (Richards, 2005). This matrix, which is made during cumulus expansion and which surrounds the oocyte, is similar to the matrix generated at sites of tissue injury (Richards et al., 2008). Cumulus expansion is also dependent on *PTGS2* and *PTX3,* which stabilises the hyaluronan matrix (Richards et al., 2008) and, interestingly, also facilitates macrophage pathogen recognition through Toll-like receptors (Bottazzi et al., 2006).

An important paper related to the present study was published by Liu *et al* in 2009 and examined the effect of IL-6 on murine COCs (Liu et al., 2009). This paper reported that expression of *Il6* is up-regulated just before ovulation and, *in vitro,* can be induced by FSH, EGF-like amphiregulin or PGE_2. This *in vitro* up-regulation was blocked using inhibitors for p38 MAPK, MEK or PKA. However, perhaps more interesting was the finding that IL-6 is capable of inducing murine COC expansion, also increasing expression of *Ptgs2, Has2* and *Ptx3.* In previous chapters, it was reported that IL-6 is produced by GCs in response to challenge with bacterial PAMPs, which suggests PAMPs could cause inappropriate expansion of the COC and oocyte activation. This hypothesis was tested in a recent paper, which treated COCs with LPS during *in vitro* maturation and found that LPS increased expansion of COCs and perturbed oocyte meiosis (Bromfield and Sheldon, 2011).

Previous investigations into the effect of PAMPs on oocyte health have focussed on the COC. However, of greater interest is how exposure to PAMPs or cytokines within the ovarian follicle during maturation may affect the subsequent embryo. Due to difficulties in forming an embryo, and not wanting to introduce LPS in semen to the investigations (Teankum et al., 2007, Fujita et al., 2011), the current work used parthenotes as a model for embryos. During parthenogenesis, an oocyte is activated by inducing calcium oscillations and inhibiting cytosolic protein synthesis. The activated oocyte acts as if it has been fertilised, with resumption of meiosis, pro-nuclear formation, DNA synthesis and, in cattle, division up to the blastocyst stage (Soloy et al., 1997). However, some differences in gene expression have been identified with altered expression of genes related to pregnancy (including *PLAC8, CDX2* and *GAB1*) and metabolism (including *PTGS2*) between buffalo embryos created through parthenogenesis and embryos created through IVF (Abdoon et al., 2012). A recent paper examined the effects of IL-6 on porcine parthenogenesis and identified a pro-survival effect, with increased expression of the anti-apoptotic *BIRC5* (also known as *SURVIVIN*) and decreased expression of *BCL2* and *CASP3* (Shen et al., 2012). Other genes found to be affected by IL-6 treatment included *STAT3,* which is involved in IL-6 signalling and is essential for early embryo development; *POU5F1,* which is involved in pluripotency, is necessary for embryo development and interacts with the STAT3 negative regulator Sox2, and *IL6ST,* whose protein product forms part of the IL-6 receptor. As it was previously shown that GCs produce IL-6 in response to LPS or PAM (chapters 3 and 4), these genes were of interest in the current study.

The hypothesis for this chapter was that the presence of PAMPs during IVM perturbs COC expansion and parthenogenesis. The aims of this chapter were to investigate whether COCs exhibit an innate immune response to PAMP treatment during IVM and whether PAMP treatment affects cumulus expansion, oocyte maturation or early embryo development. Firstly, COCs were exposed to PAMPs during IVM and scored for expansion. The accumulation of pro-inflammatory cytokines and chemokines was measured by ELISA and expression of appropriate genes estimated by multiplex PCR. After IVM in the presence of LPS or PAM, oocytes were activated and cleavage noted over 42 h. After this, expression of key genes involved in early embryogenesis was estimated by multiplex PCR.

5.2 Methods

5.2.1 Cumulus-oocyte complex *in vitro* maturation

Cumulus-oocyte complexes (COCs) were pooled from 12 to 25 ovaries and washed twice in collection medium without heparin, as described in general methods (chapter 2). Healthy COCs with at least 3 layers of cumulus cells were cultured for 3 h or 24 h in one of 6 treatment groups: i) control IVM medium, ii) IVM medium containing 1 μg/ml LPS, iii) IVM medium containing 1 μg/ml PAM, iv) IVM medium containing 2.5 μg/ml FSH and 10 μg/ml LH, v) IVM medium containing 2.5 μg/ml FSH, 10 μg/ml LH and 1 μg/ml LPS or vi) IVM medium containing 2.5 μg/ml FSH, 10 μg/ml LH and 1 μg/ml PAM. After 3 h, COCs were lysed with buffer RLT for analysis by multiplex PCR. After 24 h, COCs were scored for expansion, supernatants were collected and COCs were lysed with buffer RLT for analysis of gene expression.

5.2.2 Parthenogenesis and culture of parthenotes

After 24 h IVM, oocytes were stripped of their cumulus cells using hyaluronidase and manual pipetting. After 3 washes, the oocytes were activated using ionomycin and mitosis induced using DMAP. After a further 3 washes, oocytes were cultured for 42 h, before being lysed in 75 μl buffer RLT for analysis of gene expression by multiplex PCR. Cell number was recorded at 22 h and 42 h; these time points were chosen because they are similar to those used in previous investigations into oocyte cleavage and bovine parthenotes activated with the same protocol as used in the present work show no difference in cleavage between 42 h and 48 h (Susko-Parrish et al., 1994, Ross et al., 2008, Somfai et al., 2010, Liang et al., 2011).

5.2.3 ELISA

Accumulation of IL-6 and IL-8 was measured in the supernatant of COCs treated with LPS or PAM, as described in general methods (chapter 2), using a kit from Thermo Fisher Scientific (IL-6) and the in-house sensitive IL-8 kit. Two technical replicates were used for each sample in each ELISA, from least 4 experiments.

5.2.4 RNA extraction, cDNA synthesis and PCR

The COCs were treated with control medium or medium containing 1 μg/ml LPS or 1 μg/ml PAM for 3 h or 24 h. Cells were washed once in PBS and lysed using 75 μl (for ≤ 12 COCs) or 350 μl (for ≥ 13 COCs) buffer RLT. Parthenotes were collected after 42 h culture and lysed using 75 μl buffer RLT. The cells were homogenised using a Qiashredder spin column and RNA was extracted using the RNeasy Micro kit, as described in general methods. Total RNA was quantified using a Nanodrop spectrophotometer and 1 μg total RNA used to synthesis cDNA, using the QuantiTect kit, as described in general methods. For COCs, expression of mRNA encoding genes considered as markers of cumulus expansion (*HAS2, PTX3)* or oocyte competence (*NLRP5, ZAR1, GDF9*) was measured by multiplex PCR, using 2 technical replicates for each sample from at least 4 experiments. For parthenotes, expression of mRNA encoding genes associated with apoptosis (*BIRC5, BCL2, CASP3)*, the placenta (*GAB1, CDX2, PLAC8*), IL-6 *(STAT3, POU5F1, Il6ST)*, *DNMT1, H19* or *DUSP1* was measured by multiplex PCR, using 2 technical replicates for each sample from at least 4 experiments. Data was normalised to *L19* for both COCs and parthenotes.

5.2.5 Statistical Analyses

Data are presented as mean + SEM, with statistical analyses performed using SPSS (version 16, SPSS Inc) and significance ascribed when $P < 0.05$. Categorised data (COC expansion and parthenote cleavage) were examined using Fisher's Exact Test. Data examining protein accumulation by ELISA were not normally distributed thus comparisons were made between treatment and control by Mann Whitney U test. Multiplex data were analysed using the ddCT method described in chapter 2 and error estimated as previously described (Livak and Schmittgen, 2001). Error was estimated by calculating the standard error of CT values for samples and standards and adding this error to the mean dCT values to calculate an upper and lower limit for ddCT and fold increase. The error of the fold increases was then estimated by dividing the difference between the upper and lower limit by the Z value 3.92. Data were then analysed using the ANOVA function in GraphPad Prism.

5.3 Results

5.3.1 Exposure of COCs to PAMPs perturbed *in vitro* maturation

Previously, it was reported that exposure of COCs to IL-6 or LPS increased cumulus expansion and perturbed oocyte meiosis during *in vitro* maturation (Liu et al., 2009, Bromfield and Sheldon, 2011). Therefore, the present study examined the effects of LPS and PAM on cumulus expansion and gene expression. The COCs were cultured for 24 h in control medium or medium containing LPS or PAM, in the presence or absence of gonadotrophins.

As expected, treatment of COCs with gonadotrophins increased the percentage of COCs at stage 3 or 4 and decreased the percentage of COCs at stage 0 or 1 (with gonadotrophin: 76% at stage 3 or 4; 8% at stage 0 or 1 vs 7% at stage 3 or 4; 73% at stage 0 or 1 without gonadotrophin; $P < 0.001$), showing that the COCs used were healthy and responded appropriately to gonadotrophin treatment. Challenging the COCs with 10 μg/ml LPS in the presence of gonadotrophins increased COC expansion to stage 4 (72% vs 45% $P < 0.001$), but also increased the number which were dead or had no response (Fig 5.1, 5.3; 12% vs 3%; $P < 0.05$). However, LPS had no significant effect on expansion when COCs were cultured in gonadotrophin-free medium (Fig 5.1, 5.3; $P > 0.1$). Treatment of COCs with 1 μg/ml PAM in the presence of gonadotrophins decreased COC expansion to stage 4 (Fig 5.2-5.3; 26% vs 45%; $P < 0.05$). However, there was no effect of 10 μg/ml PAM treatment on COC expansion ($P < 0.6$). Treatment of COCs with 10 μg/ml PAM increased the number which were dead or had no response (Fig 5.2-5.3; $P < 0.05$).

5.3.2 Treatment of COCs with PAM during IVM increased accumulation of inflammatory mediators

After 24 h IVM, there was no significant effect of LPS on COC accumulation of IL-6 or IL-8 (Fig 5.4A, C). In contrast, supernatants collected from COCs treated with PAM increased accumulation of IL-6 (Fig 5.4B) and tended towards increased accumulation of IL-8 (Fig 5.4D).

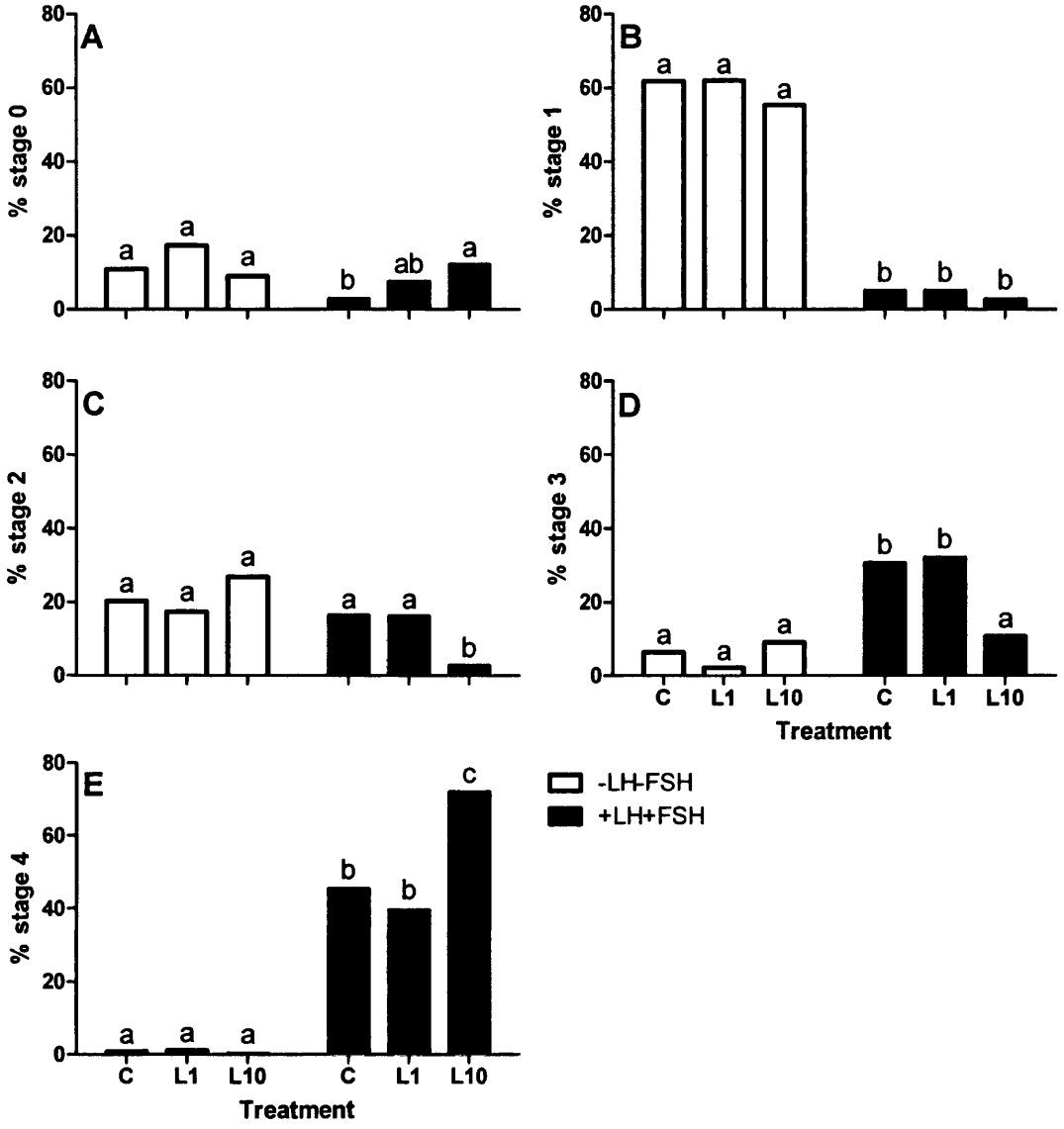


Figure 5.1 Treatment with LPS increased bovine cumulus expansion. Bovine COCs were isolated from emerged follicles and matured *in vitro* in control medium ('C') or medium containing 1 ('L1') or 10 μg/ml LPS ('L10') in the absence (open bars) or presence (black bars) of 2.5 μg/ml FSH and 10 μg/ml LH. After 24 h, cumulus expansion was scored from 0 (A) to 4 (E), with 4 being most expanded. Data presented as percentage of COCs at each stage of expansion, based on data from at least 6 experiments and expressed as a percentage of the total COCs treated. Data differing by Fisher's Exact Test are denoted with different letters.

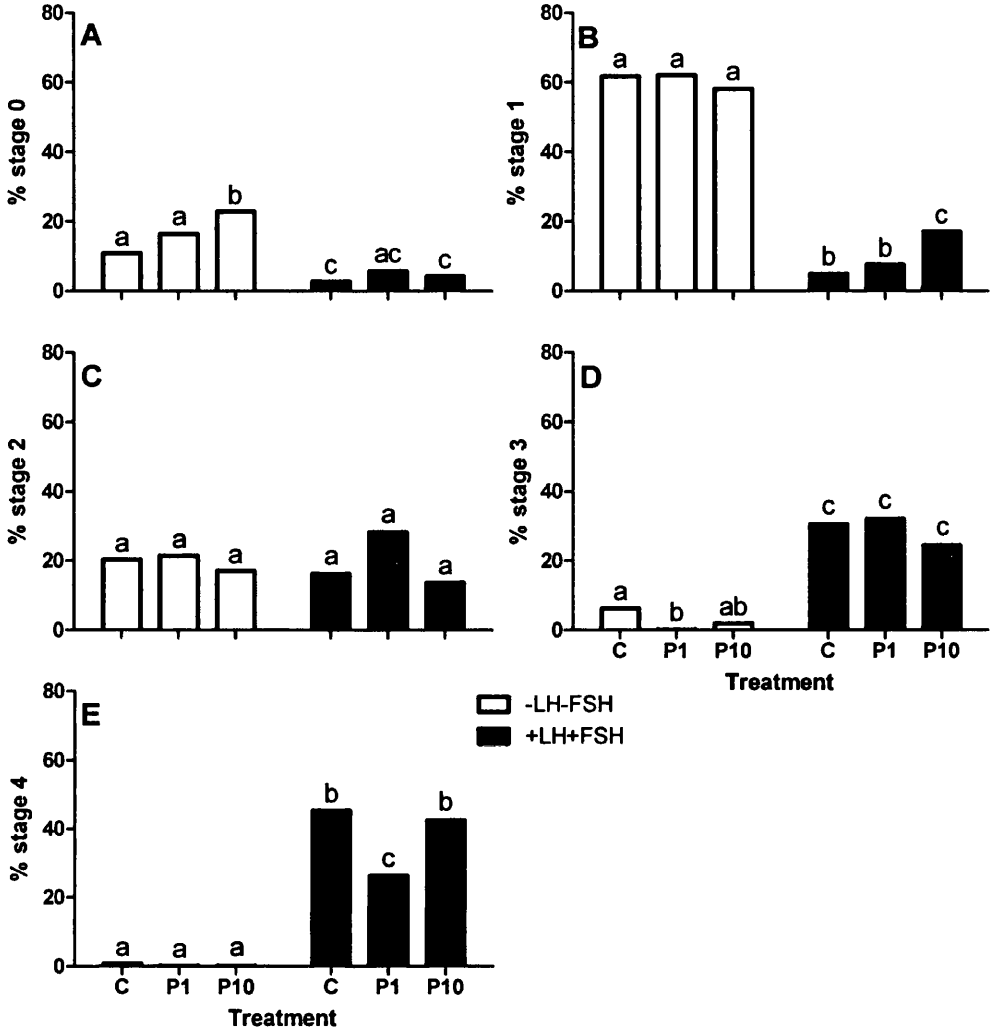


Figure 5.2 Treatment with PAM decreased bovine cumulus expansion. Bovine COCs were isolated from emerged follicles and matured *in vitro* in control medium ('C') or medium containing 1 ('P1') or 10 μg/ml PAM ('P10') in the absence (open bars) or presence (black bars) of 2.5 μg/ml FSH and 10 μg/ml LH. After 24 h, cumulus expansion was scored from 0 (A) to 4 (E), with 4 being most expanded. Data presented as percentage of COCs at each stage of expansion, based on data from at least 5 experiments and expressed as a percentage of the total COCs treated. Data differing by Fisher's Exact Test are denoted with different letters.

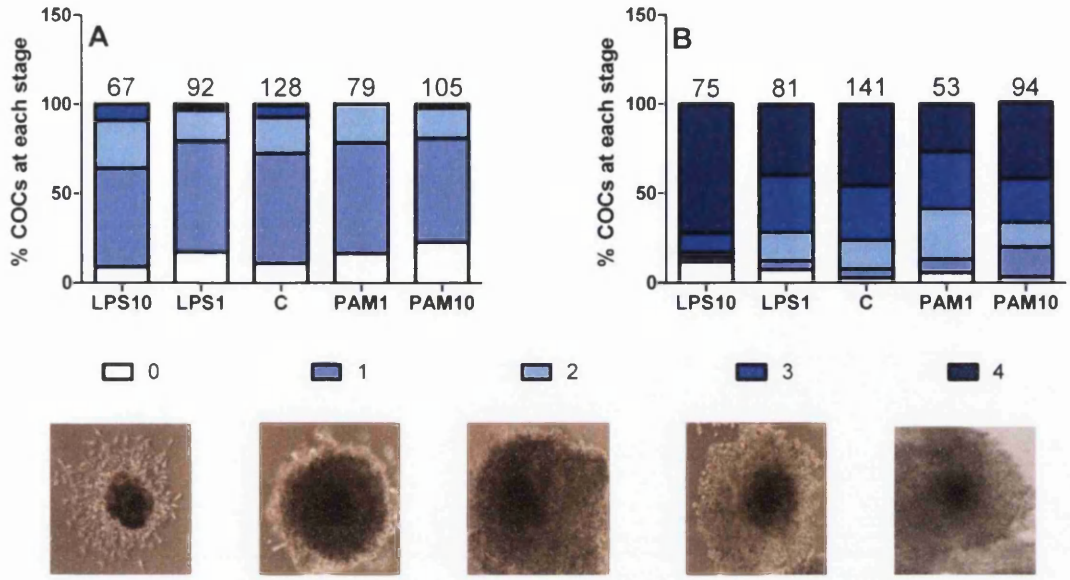


Figure 5.3 The presence of PAMPs during IVM affected cumulus expansion. Bovine COCs were isolated from emerged follicles and matured *in vitro* in control medium ('C') or medium containing 1 or 10 μg/ml LPS or PAM in the absence (A) or presence (B) of 2.5 μg/ml FSH and 10 μg/ml LH. After 24 h, cumulus expansion was scored from 0 to 4 (with 4 being most expanded). Data presented as percentage of COCs at each stage of expansion, based on data from at least 5 experiments and expressed as a percentage of the total COCs treated in each group. The number of COCs in each treatment group is indicated above each bar. Representative images of each stage are shown below the key.

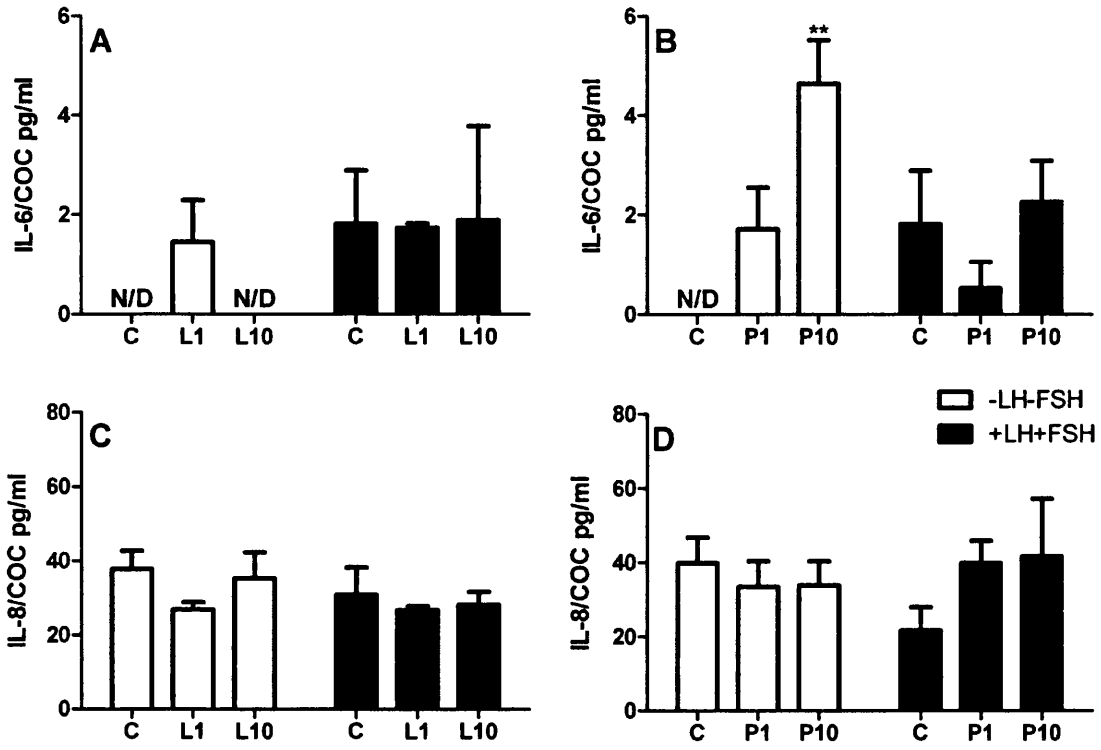


Figure 5.4 Treatment of COCs with PAM during IVM increased accumulation of IL-6. Bovine COCs were isolated from emerged follicles and matured *in vitro* in control medium ('C') or medium containing 1 or 10 μg/ml LPS ('L1' or 'L10') or PAM ('P1' or 'P10') in the absence (open bars) or presence (closed bars) of 2.5 μg/ml FSH and 10 μg/ml LH. After 24 h, supernatants were collected and the accumulation of IL-6 (A, B) and IL-8 (C, D) measured by ELISA. Data expressed as pg/ml cytokine production per COC and are presented as mean + SEM from at least 3 replicates. Values differ from control within gonadotrophin group by Mann Whitney, $P < 0.05$. N/D = below limits of detection.

5.3.3 Exposure of COCs to PAMPs perturbed expression of genes associated with cumulus expansion and oocyte quality

As shown in Fig 5.4, treatment of COCs with PAM increased accumulation of IL-6. As IL-6 increases murine expression of *Has2* and *Ptx3* (Liu et al., 2009), the present study aimed to address whether treatment of COCs with PAMPs perturbs the expression of these genes associated with cumulus expansion or the expression of maternal effect genes associated with oocyte quality.

Treatment of COCs with LPS for 3 h decreased expression of *PTX3* and *HAS2*, when cells were treated with or without gonadotrophins (Fig 5.5A, B). Conversely, treatment of COCs with 10 μg/ml PAM for 3 h increased expression of *PTX3* and *HAS2* (Fig 5.5C, D). Treatment with either LPS (Fig 5.6) or PAM (Fig 5.7) for 3 h decreased expression of *NLRP5, ZAR1* and *GDF9*.

After 24 h treatment with PAMPs during IVM, COCs increased expression of *PTX3* in response to 10 μg/ml LPS (Fig 5.8A) and decreased expression of *HAS2* (Fig 5.8B) in response to either 1 or 10 μg/ml LPS. Treatment of COCs in gonadotrophin-free medium containing PAM increased expression of *PTX3* and *HAS2* (Fig 5.8C, D). However, in medium containing gonadotrophin, 1 μg/ml PAM decreased expression of *HAS2* (Fig 5.8D). After 24 h treatment with LPS, COCs decreased accumulation of *NLRP5* (Fig 5.9A), *ZAR1* (Fig 5.9B) and *GDF9* (Fig 5.9C). In contrast, PAM treatment increased expression of all 3 maternal effect genes (Fig 5.10).

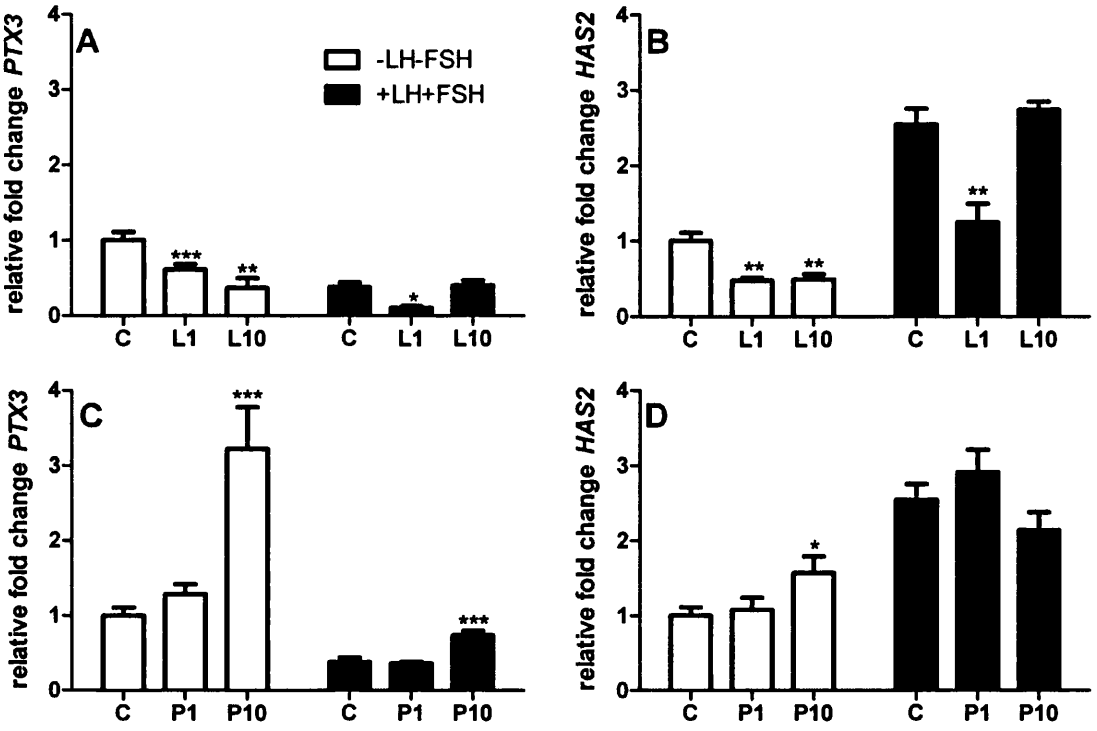


Figure 5.5 Treatment of COCs for 3 h with LPS decreased expression of genes involved with cumulus expansion; PAM treatment increased expression of these genes. Bovine COCs were isolated from emerged follicles and matured *in vitro* in control medium ('C') or medium containing 1 or 10 μg/ml LPS ('L1' or 'L10') or PAM ('P1' or 'P10') in the absence (open bars) or presence (closed bars) of 2.5 μg/ml FSH and 10 μg/ml LH. After 3 h culture, COCs were lysed, RNA extracted and the expression of *PTX3* (A, C) and *HAS2* (B, D) estimated by multiplex PCR. Data represented as mean plus estimated error, relative to control, from at least 4 independent experiments and differ from control within group by ANOVA, followed by Dunnett's multiple comparison post-hoc T-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

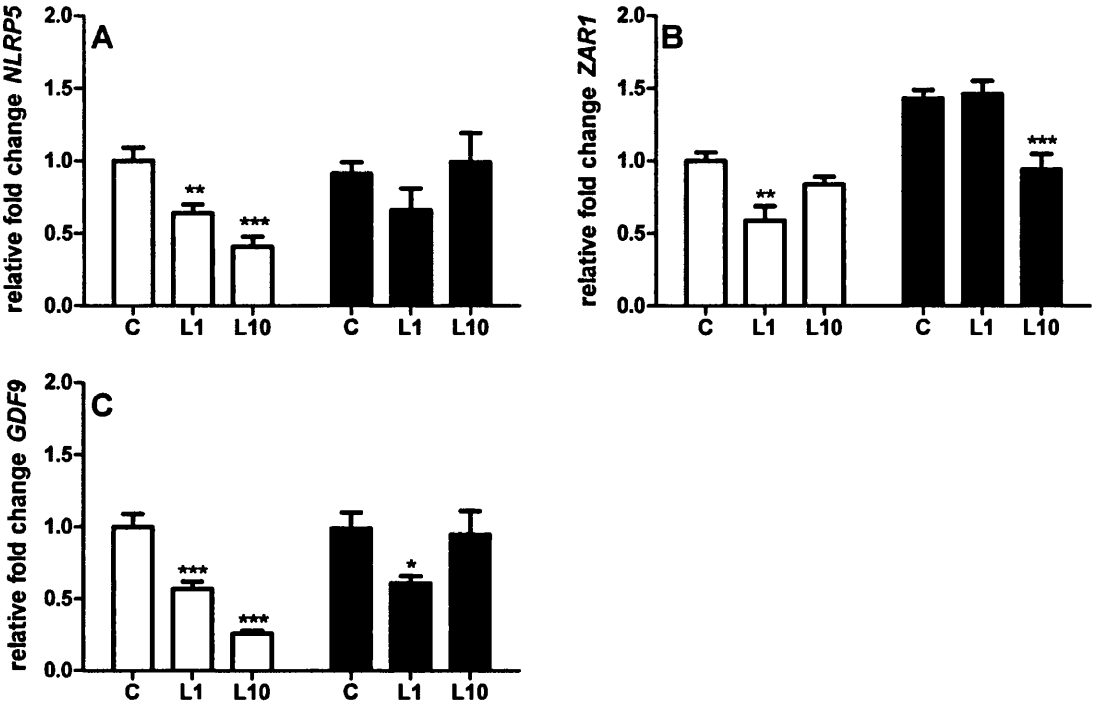


Figure 5.6 Treatment of COCs for 3 h with LPS decreased expression of maternal effect genes associated with oocyte quality. Bovine COCs were isolated from emerged follicles and matured *in vitro* in control medium ('C') or medium containing 1 or 10 μg/ml LPS ('L1' or 'L10') in the absence (open bars) or presence (closed bars) of 2.5 μg/ml FSH and 10 μg/ml LH. After 3 h culture, COCs were lysed, RNA extracted and the expression of *NLRP5* (A), *ZAR1* (B) and *GDF9* (C) estimated by multiplex PCR. Data represented as mean plus estimated error, relative to control, from at least 4 independent experiments and differ from control within group by ANOVA, followed by Dunnett's multiple comparison post-hoc T-test, ** $P < 0.01$, *** $P < 0.001$

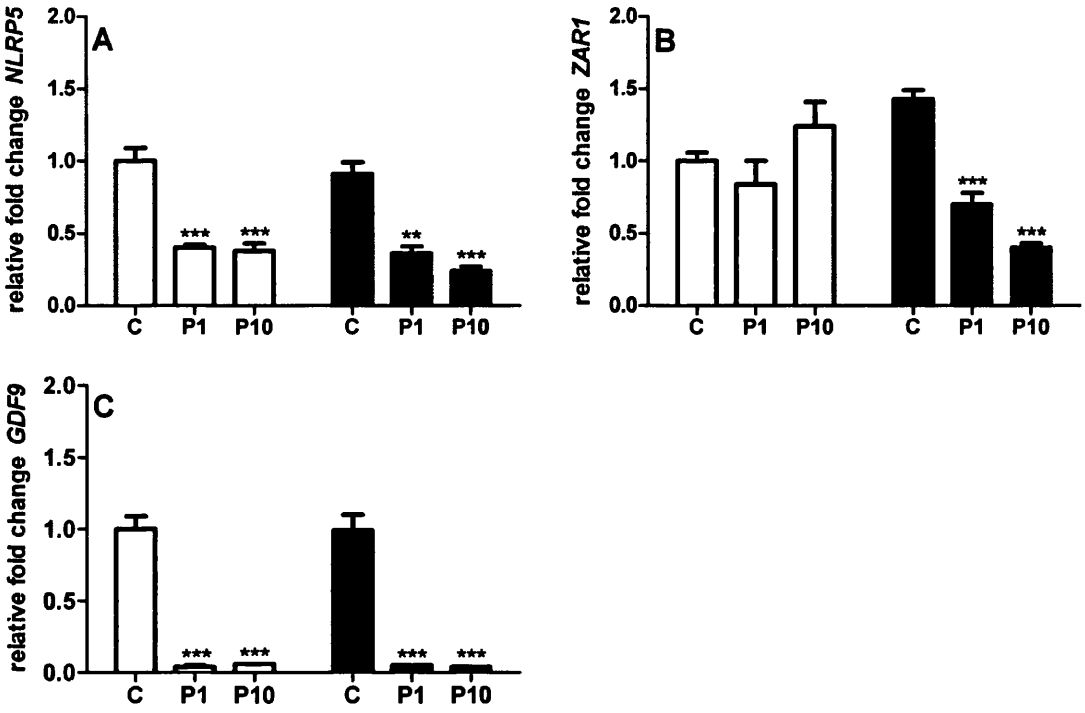


Figure 5.7 Treatment of COCs for 3 h with PAM decreased expression of maternal effect genes associated with oocyte quality. Bovine COCs were isolated from emerged follicles and matured *in vitro* in control medium ('C') or medium containing 1 or 10 μg/ml PAM ('P1' or 'P10') in the absence (open bars) or presence (closed bars) of 2.5 μg/ml FSH and 10 μg/ml LH. After 3 h culture, COCs were lysed, RNA extracted and the expression of *NLRP5* (A), *ZAR1* (B) and *GDF9* (C) estimated by multiplex PCR. Data represented as mean plus estimated error, relative to control, from at least 5 independent experiments and differ from control within group by ANOVA, followed by Dunnett's multiple comparison post-hoc T-test, ** $P < 0.01$, *** $P < 0.001$

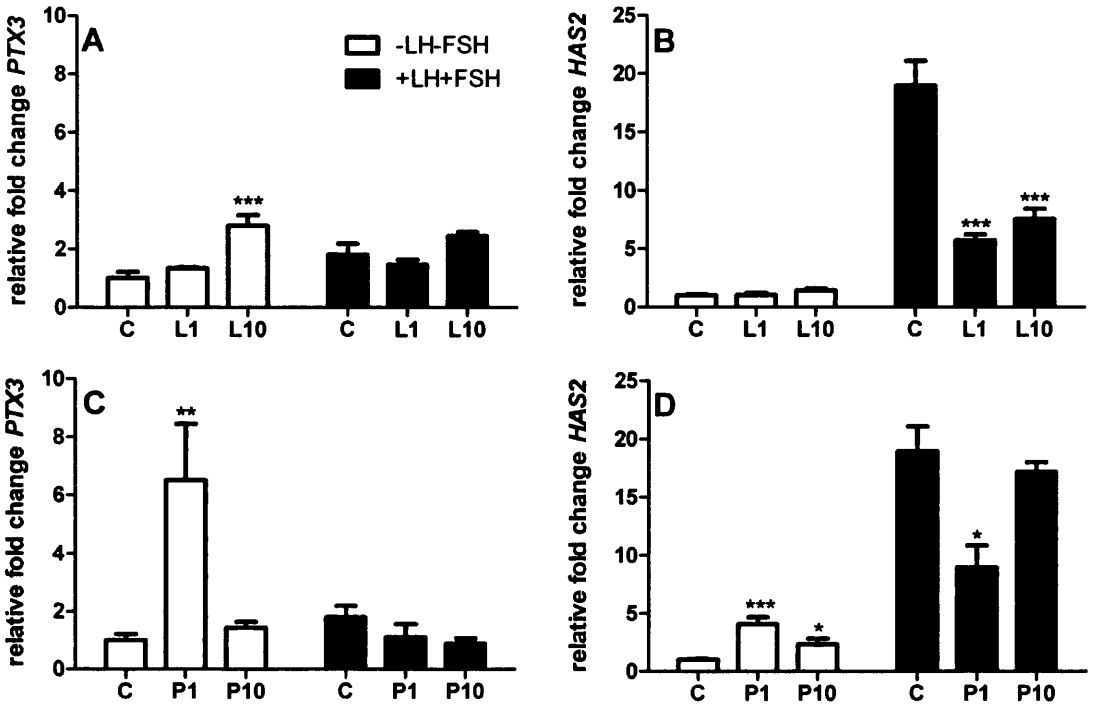


Figure 5.8 Treatment of COCs for 24 h with LPS or PAM impacted expression of genes involved with cumulus expansion. Bovine COCs were isolated from emerged follicles and matured *in vitro* in control medium ('C') or medium containing 1 or 10 μg/ml LPS ('L1' or 'L10') or PAM ('P1' or 'P10') in the absence (open bars) or presence (closed bars) of 2.5 μg/ml FSH and 10 μg/ml LH. After 24 h culture, COCs were lysed, RNA extracted and the expression of *PTX3* (A, C) and *HAS2* (B, D) estimated by multiplex PCR. Data represented as mean plus estimated error, relative to control, from at least 4 independent experiments and differ from control within group by ANOVA, followed by Dunnett's multiple comparison post-hoc T-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

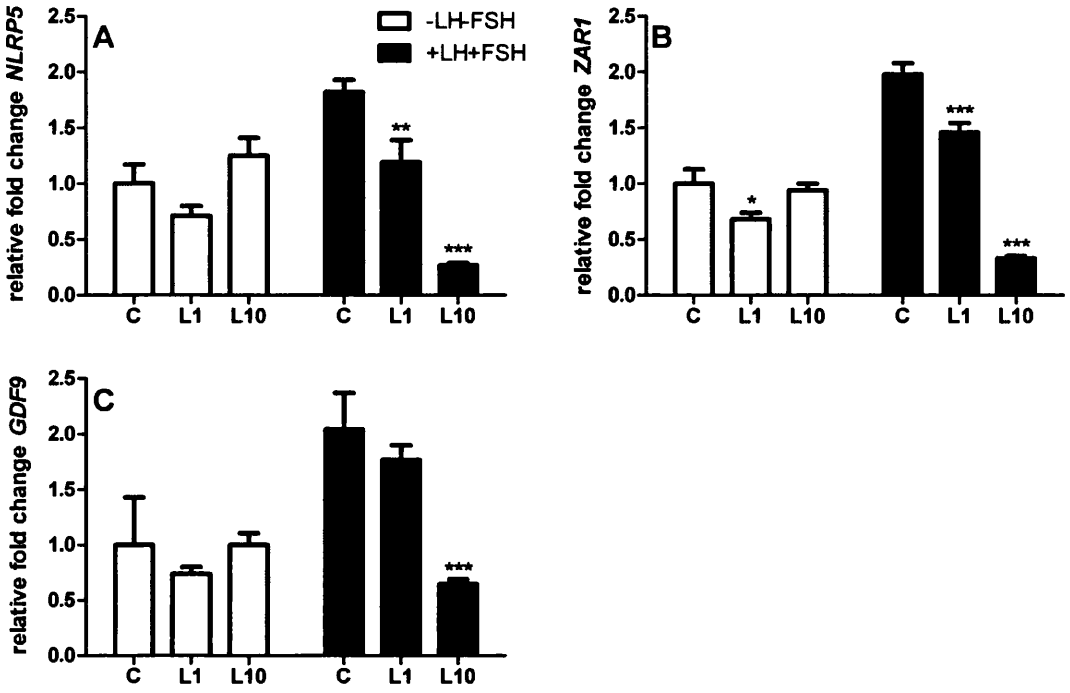


Figure 5.9 Treatment of COCs for 24 h with LPS decreased expression of maternal effect genes associated with oocyte quality. Bovine COCs were isolated from emerged follicles and matured *in vitro* in control medium ('C') or medium containing 1 or 10 μg/ml LPS ('L1' or 'L10') in the absence (open bars) or presence (closed bars) of 2.5 μg/ml FSH and 10 μg/ml LH. After 24 h culture, COCs were lysed, RNA extracted and the expression of *NLRP5* (A), *ZAR1* (B) and *GDF9* (C) estimated by multiplex PCR. Data represented as mean plus estimated error, relative to control, from at least 4 independent experiments and differ from control within group by ANOVA, followed by Dunnett's multiple comparison post-hoc T-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

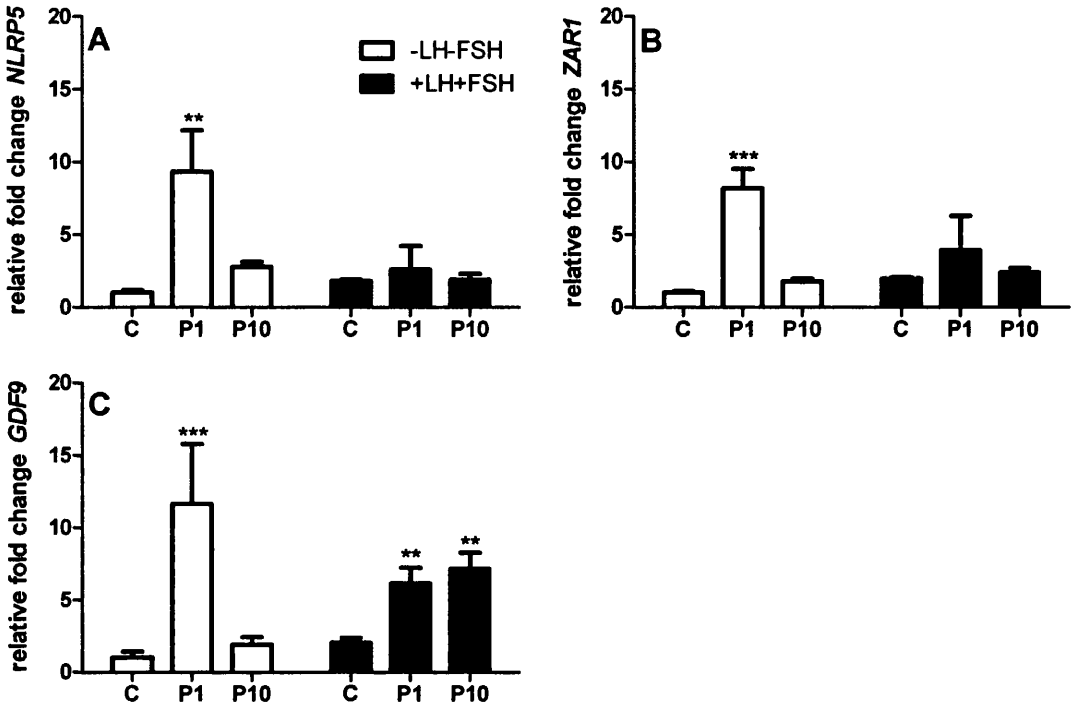


Figure 5.10 Treatment of COCs for 24 h with PAM increased expression of maternal effect genes associated with oocyte quality. Bovine COCs were isolated from emerged follicles and matured *in vitro* in control medium ('C') or medium containing 1 or 10 μg/ml PAM ('P1' or 'P10') in the absence (open bars) or presence (closed bars) of 2.5 μg/ml FSH and 10 μg/ml LH. After 24 h culture, COCs were lysed, RNA extracted and the expression of *NLRP5* (A), *ZAR1* (B) and *GDF9* (C) estimated by multiplex PCR. Data represented as mean plus estimated error, relative to control, from at least 4 independent experiments and differ from control within group by ANOVA, followed by Dunnett's multiple comparison post-hoc T-test, ** $P < 0.01$, *** $P < 0.001$

5.3.4 Exposure of COCs to PAMPs during IVM increased parthenote cleavage rate

In the present study, the effect of bacterial infection on ovarian health prior to ovulation was of interest. Therefore, COCs were matured *in vitro* in control medium or medium containing LPS or PAM before activating with ionomycin and DMAP. The oocytes which had undergone IVM in the presence of LPS tended towards increased cleavage rate after 22 h (Fig 5.11A; 20% vs 12%; $P < 0.06$). After 42 h, IVM with either LPS or PAM increased cleavage rate (Fig 5.12B; $P < 0.01$).

5.3.5 Exposure of COCs to PAMPs during IVM perturbed expression of genes necessary for normal embryo development

Cumulus-oocyte complexes were treated with PAMPs during IVM, activated, cultured for 42 h and the expression of genes necessary for normal embryo development measured by multiplex PCR. The expression of *ZAR1* (Fig 5.13A) and *GDF9* (Fig 5.13B) was increased by treatment with PAM. Treatment with LPS tended towards increased expression of *GDF9* (Fig 5.13B; $P < 0.06$).

Treatment with PAM decreased expression of *CASP3* (Fig 5.14A) but there was no significant effect of LPS or PAM treatment on expression of *DUSP1* (Fig 5.14B) or *GAB1* (Fig 5.14C). Although primers for *NLRP5, BCL2, BIRC5, CDX2, PLAC8, STAT3, POU5F1, H19* and *DNMT1* worked with positive control cDNA, expression of these genes in COC samples could not be detected except *STAT3, BIRC5* and *H19* could be detected in 3 out of 5 samples treated with PAM.

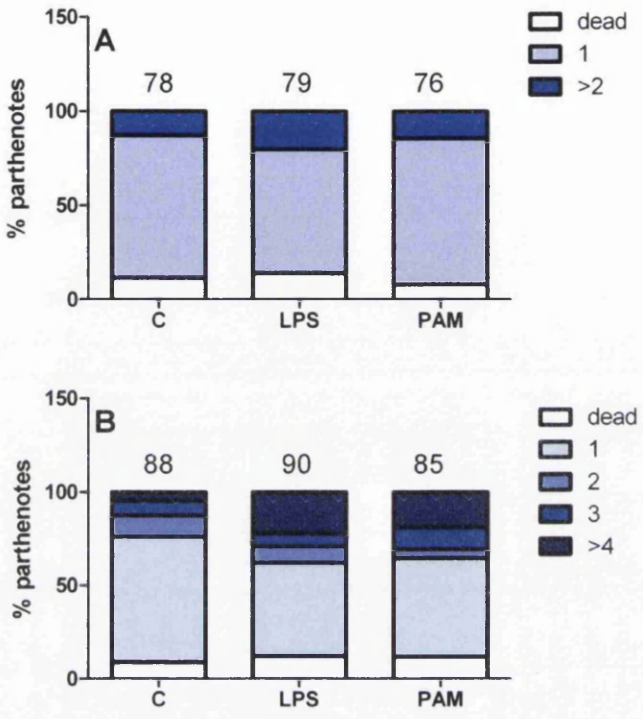


Figure 5.11 The presence of PAMPs during IVM increased cleavage of activated oocytes during parthenogenesis. Bovine COCs were isolated from emerged follicles and matured *in vitro* in medium containing 2.5 μg/ml FSH and 10 μg/ml LH ('C') or 10 μg/ml LPS or PAM in gonadotrophin-containing medium. After 24 h, COCs were stripped with hyaluronidase and activated using ionomycin and DMAP. After 22 h (A) and 42 h (B) culture in embryo culture medium, the number of cells in each parthenote were counted. Data presented as percentage of parthenotes with 1, 2, 3 or > 4 cells, based on data from at least 5 experiments. The number of parthenotes in each treatment group is indicated above each bar.

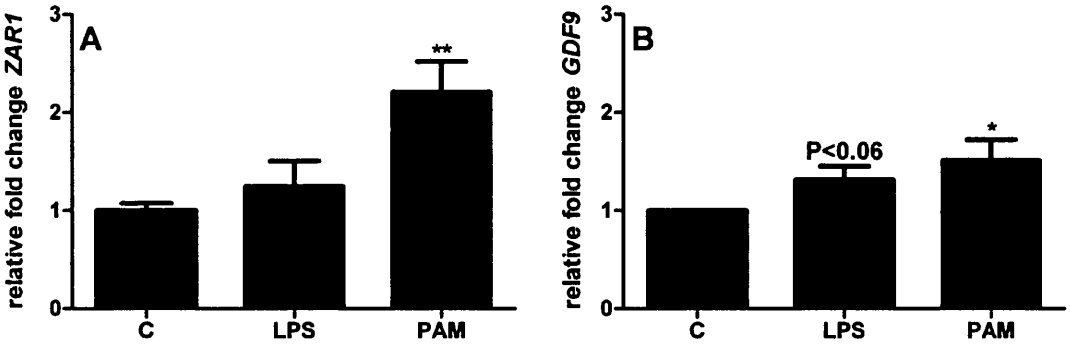


Figure 5.12 *In vitro* maturation of COCs in the presence of PAMPs increased parthenote expression of maternal effect genes associated with oocyte quality. Bovine COCs were isolated from emerged follicles and matured *in vitro* in medium containing 2.5 μg/ml FSH and 10 μg/ml LH ('C') or 10 μg/ml LPS or PAM in gonadotrophin-containing medium. After 24 h, COCs were stripped with hyaluronidase and activated using ionomycin and DMAP. After a further 42 h culture in embryo culture medium, RNA was extracted and the expression of *ZAR1* (A) and *GDF9* (B) estimated by multiplex PCR. Data represented as mean plus estimated error, relative to control, from 5 independent experiments and differ from control within group by ANOVA, followed by Dunnett's multiple comparison post-hoc T-test, * $P < 0.05$, ** $P < 0.01$

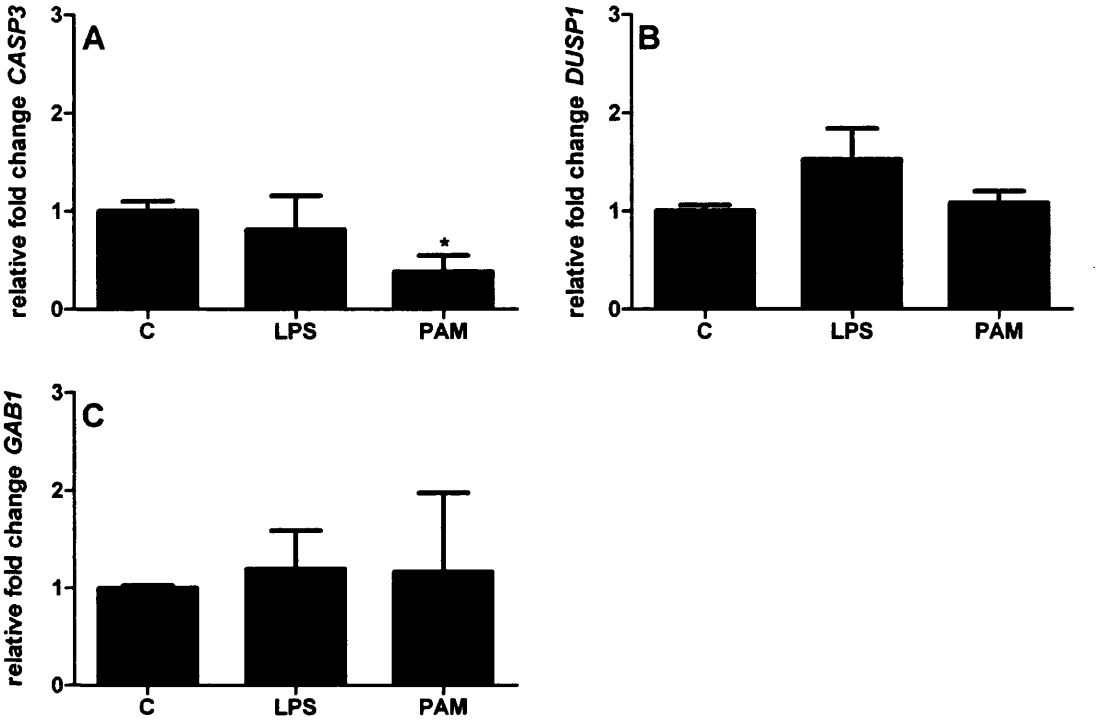


Figure 5.13 *In vitro* maturation of COCs in the presence of PAMPs had little effect on parthenote expression of genes associated with apoptosis or placenta development. Bovine COCs were isolated from emerged follicles and matured *in vitro* in medium containing 2.5 μg/ml FSH and 10 μg/ml LH ('C') or 10 μg/ml LPS or PAM in gonadotrophin-containing medium. After 24 h, COCs were stripped with hyaluronidase and activated using ionomycin and DMAP. After a further 42 h culture in embryo culture medium, RNA was extracted and the expression of *CASP3* (A) *DUSP1* (B) and *GAB1* (C) estimated by multiplex PCR. Data represented as mean plus estimated error, relative to control, from 5 independent experiments and differ from control within group by ANOVA, followed by Dunnett's multiple comparison post-hoc T-test, * $P < 0.05$, ** $P < 0.01$

5.4 Discussion

Bacterial infections of the uterus or mammary gland perturb ovarian follicle growth, endocrine function and ovulation (Sheldon et al., 2002, Lavon et al., 2011a, Lavon et al., 2011b). Lipopolysaccharide has been found in the follicular fluid from follicles of cows with uterine disease (Herath et al., 2007) and bovine GCs respond to LPS, producing IL-6 (Bromfield and Sheldon, 2011). Interleukin-6 causes murine cumulus expansion and increases expression of genes involved in this cumulus expansion and oocyte maturation (Liu et al., 2009). So, the present study considered whether the bovine oocyte and subsequent embryo are affected by the presence of PAMPs in follicular fluid. The presence of LPS or PAM during IVM perturbed cumulus expansion and increased production of inflammatory mediators by the COCs. The expression of genes involved in maturation including *HAS2* and *PTX3* was perturbed after 3 h or 24 h treatment with either PAMP. The expression of maternal effect genes associated with oocyte quality (*NLRP5, ZAR1, GDF9*) was decreased after 3 h or 24 h treatment with LPS or PAM. Interestingly, the presence of PAMPs during IVM increased cleavage rate and increased expression of *ZAR1* and *GDF9* in the subsequent parthenote. However, there was little effect of PAMPs on expression of genes involved in apoptosis or placental development, except for expression of *GDF9,* which was increased after 24 h PAM treatment. The perturbation of oocyte maturation and early embryogenesis by PAMPs provides the basis for understanding how infections distal to the ovary may affect oocyte health and why a healthy embryo may not develop despite ovulation occurring.

To the best of the author's knowledge, this is the first study examining the effects of pathogen-associated molecular patterns on oocyte gene expression and development. A recent paper investigated the effects of LPS exposure during IVM on bovine oocyte cumulus expansion and maturation and found that LPS increased accumulation of IL-6 by COCs, increased rates of cumulus expansion in the absence of FSH and increased rates of meiotic failure (Bromfield and Sheldon, 2011). In contrast, the present study reported that LPS had a significant effect on cumulus expansion in the presence, but not the absence of gonadotrophin. In the 2011 study, around 90% of COCs expanded in the presence of gonadotrophin. In contrast, in the present study, only 50% of COCs expanded in the presence of gonadotrophin and

absence of LPS. Regardless, both studies showed that LPS perturbed cumulus expansion. However, further experiments are necessary to determine the magnitude of the effect of LPS. Potentially, it is an interaction between LH and LPS which increases COC expansion, decreasing the role of FSH. Supporting the theory of crosstalk between innate immune signalling pathways and the FSH-receptor pathway, FSHR is required for innate immunity in *Caenorhabditis elegans* and it was previously hypothesised that the FSH-receptor may play a role in granulosa pathogen response (Powell et al., 2009). Is it possible that LPS may signal through the FSH receptor, perhaps binding in concert with LH? Supporting this theory, ovulatory concentrations of LH can activate FSHR in fish, although this is less common in mammals (Andersson et al., 2009). Potentially, LPS increases the affinity of the FSHR for LH. An alternative theory is that LPS increases accumulation of IL-6 which then causes COC expansion, as it has previously been shown to do in mouse (Liu et al., 2009). However, in the present study, PAM rather than LPS increased accumulation of IL-6, but did not increase cumulus expansion.

The present study reported that LPS decreased and PAM increased expression of *HAS2* and *PTX3* necessary for cumulus expansion when COCs were treated for 3 h. After 24 h, expression of *PTX3* was increased by either LPS or PAM treatment. However, the effect of 24 h PAMP treatment on expression of *Has2* varied depending on whether COCs were cultured with or without gonadotrophin, with LPS decreasing *HAS2* expression stimulated by gonadotrophin and PAM increasing *HAS2* expression of COCs in gonadotrophin-free medium but decreasing *HAS2* expression when using gonadotrophin-containing medium. These results were unexpected because LPS increased expansion but mostly decreased expression of genes associated with expansion, suggesting that LPS may trigger expansion through atypical pathways. A link between hyaluronan (synthesised via *Has2*) and TLRs was previously identified when it was reported that hyaluronan binds TLR2 and TLR4 and induces expression of *Il6* in murine COCs (Shimada et al., 2008). However, these results should be explored with caution because of the difficulty in obtaining ultrapure hyaluronan free from contaminating PAMPs. It was interesting that LPS and PAM increased expression of *PTX3* because *PTX3* facilitates recognition of pathogens by macrophages (Mantovani et al., 2003) and is induced by LPS treatment of human neutrophils or macrophages (Imamura et al., 2007). Thus, potentially

PTX3 could also aid in sensing of pathogens by cumulus-oocytes complexes. This is an interesting hypothesis as Toll was originally discovered in *Drosophila* as an essential receptor for the establishment of the dorsal-ventral pattern in developing embryos (Anderson et al., 1985) and was later found to play a role in innate immunity (Lemaitre et al., 1996).

Another interesting link between innate immunity and embryo development is the Nod-like receptor family, of which *NLRP5* is a member, which are involved with intracellular sensing of PAMPs. During bovine oocyte development, *NLPR5* is first detected in oocytes from primary follicles and accumulates during oocyte growth, before depletion after fertilisation with 5-8 cell embryos only having 10% of the initial expression levels (Pennetier et al., 2006). Although embryos from *Zar1*$^{-/-}$ knockout mice mostly arrest prior to cleavage (Wu et al., 2003), bovine *ZAR1* is expressed in the oocyte and embryo until blastocyst formation, with upregulation at the 4-cell stage, suggesting that the embryo also expresses *ZAR1* (Brevini et al., 2004). Similarly to the other two maternal effect genes, *GDF9* is expressed throughout oocyte development and embryo growth, with levels falling until blastocyst formation, when there is no detectible expression (Pennetier et al., 2004). In the present study, treatment of COCs with LPS or PAM during IVM generally decreased COC expression of *NLRP5, ZAR1* or *GDF9,* although it was interesting that 24 h PAM treatment increased expression of *GDF9* in both COCs and subsequent parthenotes. Experiments using rats show that GDF9 is anti-apoptotic during follicular growth (Orisaka et al., 2006). Thus this increased expression of *GDF9* may be associated with cell survival. Supporting this theory, PAM decreased expression of pro-apoptotic *CASP3.* Depletion of *NLRP5* suppresses embryogenesis (Wu, 2009), thus the decrease in expression of the maternal effect genes by PAMPs may be a protective mechanism to prevent embryo development in an infected environment. However, it seems more likely that decreased *NLRP5, ZAR1* and *GDF9* expression is a carryover effect from the increased rate of maturation stimulated by PAMP treatment and could thus perturb synchronisation between embryo and uterus, and could therefore impact on establishment of pregnancy.

The present study investigated the effects of LPS and PAM on the bovine oocyte and early embryo. The presence of PAMPs during IVM is a biologically relevant problem, as cows with uterine disease collect LPS in the follicular fluid of ovarian dominant follicles (Herath et al., 2007). It was found that treatment of COCs with LPS or PAM increased cumulus expansion and accelerated oocyte development, with a knock-on effect on subsequent parthenotes developed from treated COCs, which had increased cleavage rates. Although the results published here are mainly observational, rather than mechanistic, this appears to be the first study investigating the effects of infection on expression of key genes in oocyte development. Further investigations, with a greater number of replicates, will hopefully elucidate some of the findings of this study and determine whether exposure to PAMPs during cumulus expansion and oocyte maturation affects blastocyst formation or successful embryogenesis.

Chapter 6

Toward a model for human ovarian cells

6.1 Introduction

The previous chapters report data about the impact of PAMPs on GCs, cumulus-oocyte complexes and early development of the embryo in cattle. So, the next question was whether these observations in cattle apply to other species. In trout, LPS administration has no effect on ovarian steroid production or oocyte maturation (MacKenzie et al., 2006), but leads to a significant increase in *TNF* expression in the whole ovary (Crespo et al., 2010). Avian GCs express functional TLRs and treatment with LPS has effects on their steroidogenesis (Woods et al., 2009) and expression of genes associated with inflammation, including *IL6* and *IL1B* (Abdelsalam et al., 2011). Lipopolysaccharide induces expression of *Cxcl1* and *Cxcl2* chemokines in murine GCs (Son and Roby, 2006), significantly decreases accumulation of oestradiol by LH-stimulated rat GCs (Taylor and Terranova, 1996) and increases production of IL-6 by murine GCs (Gorospe and Spangelo, 1993). The presence of LPS in culture medium during human *in-vitro* fertilisation significantly decreases incidence of pregnancy (Fishel et al., 1988) and treatment of human GCs with LPS increases expression and secretion of TNFα (Sancho-Tello et al., 1992). Pelvic inflammatory disease leads to infertility in 100,000 American women each year (N.I.A.I.D, 2009), thus it was important to investigate the effects of infection on human ovarian cell health. The most evident responses in previous chapters were by the GCs (chapters 3 & 4) rather than embryos in cattle; and working with human oocytes and embryos was not practical or ethical in the present thesis, so a human granulosa cell line was used in this chapter.

The cell line COV434 was established from a primary human granulosa cell tumour (van den Berg-Bakker et al., 1993). The COV434 cell line has several features considered essential for normal granulosa cell function, including the production and secretion of oestradiol after treatment with FSH and androstenedione; stimulation of cell growth by FSH, and communication between COV434 cells and cumulus oophorus cells containing a viable oocyte (Zhang et al., 2000). The use of a granulosa cell line to study responses to PAMPs avoids the common confounder of a mixed population of cells, including immune cells, associated with human ovarian follicle aspiration (Fedorcsák et al., 2007). However, using a cell line is less physiologically relevant as the cells are not primary cells; for example, some

apoptosis-related genes are expressed by primary GCs but not by COV434 cells (Zhang et al., 2000). It was thus necessary to validate whether the COV434 cell line was suitable for investigating the effects of infection and inflammation, in particular whether the cell line expresses pattern recognition receptors, and whether the function of the COV434 cells is perturbed by treatment with PAMPs.

Macrophages are professional immune cells and express TLRs and respond to PAMPs (Fu Qiu et al., 2011). Therefore, a human monocytic cell line was used as a positive control to verify that the techniques used were suitable. The THP-1 cell line was derived from peripheral blood of a patient with acute monocytic leukaemia (Tsuchiya et al., 1980). These cells are cultured as monocytic cells in suspension but readily differentiate into a macrophage-like cell after treatment with phorbol-12-myristate-13-acetate (PMA).

The hypothesis tested in this chapter was that the human granulosa cell line COV434 expresses TLRs and can mount an inflammatory response to pathogen associated molecules. The aim of this chapter was to investigate whether the COV434 cell line expressed TLRs at the mRNA level. Functionality of the receptors was then tested by challenging the COV434 cells with PAMPs and measuring the inflammatory response of the cells after acute exposure, at the RNA level, and also after 24 h, at the protein level. This work concentrated on two prototypical cytokines (IL-6 and IL-1β) and two prototypical chemokines (IL-8 and CCL5). The effect of PAMPs on the physiological health of the cells was also studied by examining the effect of PAMPs on oestradiol production by COV434 cells treated with FSH and androstendione. Initial work determined suitable cell culture conditions for PAMP treatment, and then the response of COV434 and THP-1 cells to PAMPs was measured at the RNA and protein level.

6.2 Methods

6.2.1 Culture of a human granulosa cell-line

The COV434 cells were cultured as described in chapter 2. After seeding cells and allowing them to equilibrate for 24 h, medium was aspirated from each well and 1 ml treatment or control medium added. After the period of time stated below, supernatants were removed and stored at -20°C, and the cells were either discarded or stored at -80°C in buffer RLT for RNA extraction. For some experiments, cells were cultured in the presence of 1 ng/ml or 100 ng/ml bovine FSH, which activates human FSH receptors (Aizen et al., 2012) and is 80% similar to human FSH (Gharib et al., 1989), and 2864.1 ng/ml androstenedione (Sigma); concentrations used previously (Zhang et al., 2000).

6.2.2 Culture of THP-1 human monocytic cell-line

The THP-1 cells were cultured as described in chapter 2. After differentiating into a macrophage phenotype using 5 ng/ml PMA, cells were washed in 0.5 ml PBS and incubated for 3 h in control THP-1 medium. Medium was aspirated from each well and 1 ml treatment or control medium added. After treatment, supernatants were removed and stored at -20°C and cells were either discarded or stored at -80°C in buffer RLT (Qiagen) for RNA analysis.

6.2.3 Treatments

The COV434 cells were first seeded at $1x10^5$ cells/ml to $4x10^6$ cells/ml ($1x10^5$, $2x10^5$, $5x10^5$, $1x10^6$, $2x10^6$, $4x10^6$ cells/ml) and challenged for 24 h with 1 μg/ml ultrapure LPS from *E. coli* O111:B4 (Invivogen, San Diego, USA) or control medium, to identify the most appropriate cell density. Subsequent experiments were carried out using a cell density of $5x10^5$ cells/ml. To explore accumulation of inflammatory mediators, the COV434 cells were treated for 0, 2, 4, 8, 24, 30 or 48 h with control media or LPS; although PAMP treatments were applied for 24 h in all subsequent experiments. Thus, for analysis of inflammatory mediator responses by mRNA or protein, the COV434 and THP-1 cells were treated for 3 or 24 h, respectively, with control medium or medium containing 1 μg/ml ultrapure LPS from *E. coli* O111:B4 (Invivogen), 1 μg/ml lipoteichoic acid (LTA; purified from *S. Aureus*; Invivogen), 1 μg/ml peptidoglycan (PGN; from *Bacillus*

subtilis; Invivogen), 1 μg/ml bacterial DNA (DNA; from *E. coli K12*; Invivogen), 0.1 μg/ml recombinant flagellin (FLA; Invivogen), 10 μg/ml hyaluronic acid (HA; Calbiochem, San Diego, USA) or 20 ng/ml human TNFα (Miltenyi Biotech, Cologne, Germany); the latter was used as a positive control because TNFα affects granulosa cell steroidogenesis and IL-6 production (Spangelo et al., 1995). In some experiments, COV434 cells were also treated with inhibitors of the TLR pathway for 24 h: 4 μM NFκB activation inhibitor Insolution, 10 μM p38 MAPK inhibitor (SB230580), 1 μM MEK inhibitor (U0126), 10 μM ERK activation inhibitor peptide 1 or 50 μM JNK inhibitor II (all Calbiochem).

6.2.4 Storage of cell lines

The COV434 cells were washed in PBS (Sigma), detached from the culture flask using 1.8 ml Accutase (Gibco) and re-suspended in 2 ml normal medium supplemented with 10% DMSO (Sigma). To freeze THP-1 cells, the suspension was centrifuged (Eppendorf 5810R, Cambridge, UK) at 1800 x g for 5 min and the supernatant discarded. The cells were then resuspended in 4 ml freezing medium (21 ml normal medium plus 3 ml DMSO (Sigma) plus 6 ml FCS (Biosera). Cell suspensions were transferred into cryovials (Elkay, Basingstoke, UK) and placed in a -1°C/min propranol-based cell freezing container (Nalgene, Roskilde, DK) for 24 h and then into a liquid nitrogen freezer.

6.2.5 Effect of treatments on cell viability, apoptosis and cytotoxicity

The COV434 cells were plated at $2x10^4$ cells/well in 100 μl medium in an opaque-walled, clear-bottomed 96-well plate (Greiner Bio One). After 24 h culture, they were treated for a further 24 h with control medium or medium containing FSH (1 or 100 ng/ml), LPS, PGN, LTA (1 μg/ml), FLA (0.1 ng/ml), TNFα (20 ng/ml), NFκB inhibitor (0.004-40 μM), p38 MAPK, ERK1/2 inhibitor (0.01-100 μM), MEK inhibitor (0.001 – 10 μM) or JNK inhibitor (0.05-500 μM). Staurosporine (10 μM for 4 h) and digitonin (30 μg/ml for 15 min) were used as positive controls. An ApoTox-Glo Triplex assay (Promega, Madison, USA) was used to assess the effect of each treatment on cell viability, apoptosis and necrosis, as described in chapter 2.

6.2.6 RNA extraction, cDNA synthesis and PCR

Expression of mRNA for *TLR1-10* was identified using the TLR RT-Primer Set (Invivogen), GoTaq Flexi DNA Polymerase kit (Promega) and dNTPs (Promega). PCR conditions were as described in the primer set instructions (Invivogen; 95 °C for 2 min, followed by 35 cycles (95°C for 30 s, 60°C for 30 s, 72°C for 2 min), followed by 72°C for 2 min and hold at 16°C). Following amplification, products were assessed on a 2% agarose gel (containing 0.8% ethidium bromide) by electrophoresis, as described in chapter 2. Images were collected using the GelDoc 2000 system and associated software (Biorad, Hemel Hempstead, UK).

Real-time quantification of *IL8, IL6* and *IL1B* relative to the reference gene *ACTB* were performed using the QuantiFast SYBR green kit (Qiagen) and the iCycler iQ thermal cycler (Biorad). PCR conditions and primer sequences are described in chapter 2.

6.2.7 ELISA

The accumulation of IL-6, IL-1β, IL-8 and CCL5 were measured in the supernatant of COV434 and THP-1 cells, using kits from R&D Systems, as described chapter 2.

Accumulation of oestradiol was measured in the supernatants of COV434 cells using an ELISA method kindly detailed by Jane Brown, Sue Walker and Karen Steinman at the Conservation and Research Centre, Smithsonian's National Zoological Park, Viriginia, USA and is a similar method to that used in the literature (Walker et al., 2002, Atsalis et al., 2004, Lueders et al., 2009, Pollock et al., 2010). The method for this ELISA is described in chapter 2.

6.2.8 Statistical analyses

Due to variation in the accumulation of cytokines in COV434 supernatants between passages, all data on COV434 cells is represented as percentage of control. For time and cell density experiments, data is shown as percentage control at the highest time or density for each culture because at 0 h or low cell density, accumulation of cytokine was sometimes below the limit of detection for the test. Significance was ascribed when $P < 0.05$ except when the Mann-Whitney U test was used, when $P \leq 0.05$ was regarded as significant. For experiments when a trend was expected (experiments using increasing cell density or culture time), the Jonckheere-Terpstra test was used, which is similar to Mann Whitney U test but for greater than 2 samples and used when a trend is expected (Bewick et al., 2004). Further details of statistical tests used are in figure legends.

6.3 Results

6.3.1 Determining the optimum cell density at which to culture COV434 cells

There does not seem to be a published consensus on the optimum cell density to grow COV434 cells, particularly with regards to measuring cytokine output. To determine this, COV434 cells were cultured at $4x10^6$, $2x10^6$, $1x10^6$, $5x10^5$, $2.5x10^5$ or $1.25x10^5$ cells/ml for 24 h in a 24-well plate, either in control medium or medium containing 1 μg/ml ultrapure LPS. There was no detectable accumulation of IL-6 or IL-1β in the supernatants of the COV434 cells, as measured by ELISA. Accumulation of CCL5 was mostly below the limit of detection for the assay, although there was some accumulation of CCL5 at the higher cell densities. Increasing cell density increased accumulation of IL-8 by cells in control medium (Fig. 6.1A). Treatment with medium containing 1 μg/ml LPS had no effect on IL-8 accumulation ($P = 0.968$). Subsequent experiments used $5x10^5$ cells/ml because this was the highest density at which the cells could be plated without medium becoming exhausted during 48 h culture. Interestingly, COV434 cells constitutively produced IL-8, although this decreased with passage number (Fig. 6.1B).

6.3.2 Determining the optimum duration for COV434 cell culture

COV434 cells were plated at $5x10^5$ cells/ml in a 24-well plate for 0, 2, 4, 8, 24, 30 or 48 h and treated with control medium or medium containing 1 μg/ml ultrapure LPS or 20 ng/ml TNFα. The accumulation of IL-8 increased over time for all treatment groups (Fig. 6.1C). There was no treatment effect for LPS ($P = 0.821$). However, there was a significant effect of TNFα treatment at 48 h ($P < 0.05$; Fig. 6.1D). For subsequent experiments, COV434 cells were cultured for 24 h because the concentration of IL-8 in the supernatants of the cells cultured for 24 h is above the limit of detection for the ELISA, yet still allows for a stimulated increase in IL-8 accumulation to be detected.

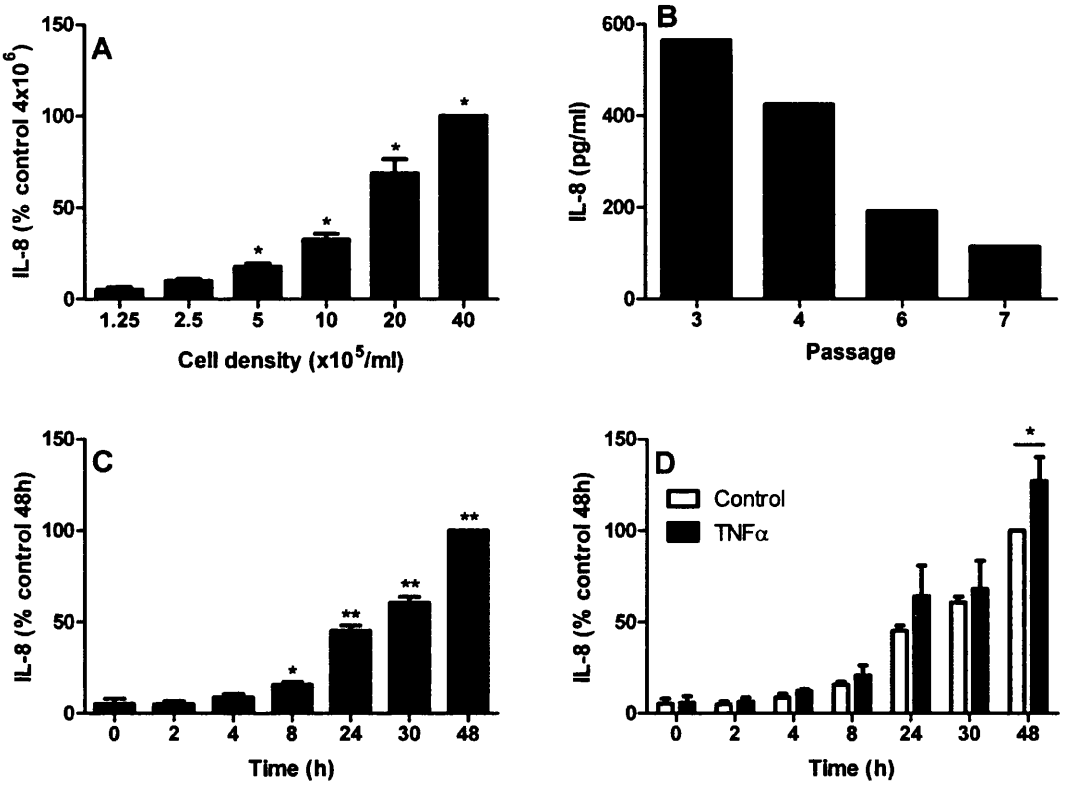


Figure 6.1 COV434 cells constitutively produced IL-8. (A) COV434 cells were cultured at $1.25x10^5$, $2.5x10^5$, $5x10^5$, $1x10^6$, $2x10^6$ or $4x10^6$ cells/ml for 24 h in control medium; supernatants were collected and the concentration of IL-8 determined by ELISA. Data are presented as mean + SEM of 3 independent experiments relative to $4x10^6$ cells/ml. Values differ from $1.25x10^5$ cells/ml by Mann-Whitney U test, * $P < 0.05$. (B) Supernatants from untreated COV434 cells were collected at passages 3, 4, 6 and 7 and the concentration of IL-8 determined by ELISA. Data are presented as mean + SEM from duplicate wells from 1 experiment. (C) COV434 cells were cultured for 0, 2, 4, 8, 24, 30 or 48 h in control medium; supernatants were collected and the concentration of IL-8 determined by ELISA. Data are presented as mean + SEM of 3 independent experiments expressed as percentage of value at 48 h. Values differ from 0 h by Mann-Whitney U test, * $P < 0.05$, ** $P < 0.01$. (D) COV434 cells were treated for 0, 2, 4, 8, 24, 30 or 48 h in control medium (□) or with 20 ng/ml TNFα (■); supernatants were collected and the concentration of IL-8 determined by ELISA. Data are presented as mean + SEM of 3 independent experiments relative to the control treatment at 48 h. Values differ between treatments by Mann-Whitney U test, * $P < 0.05$.

6.3.3 COV434 cells expressed *TLRs 4-10*

Analysis by qPCR showed that COV434 cells expressed mRNA for *TLR4-10*, but not *TLR1-3*. As expected, the THP-1 cells expressed mRNA for *TLR1-10* (Fig. 6.2).

6.3.4 Initial PAMP panel

In an initial experiment, COV434 and THP-1 cells were treated for 3 or 24 h with LPS, LTA, PGN, DNA, FLA, HA or TNFα in order to decide which treatments to carry on to further experiments. Accumulation of IL-8, IL-6, IL-1β and CCL5 was measured by ELISA and expression of *IL6, IL8* and *IL1B* was measured by qPCR. At the mRNA level, LPS, LTA, PGN and DNA increased expression of *IL1B* by COV434 cells (Fig. 6.3B) and LTA, PGN, FLA and HA increased expression of *IL6, IL1B* and *IL8* by THP-1 cells (Fig. 6.3D-F). At the protein level, only the positive control TNFα stimulated a response in COV434 cells (Fig. 6.4A-D), whereas LPS, LTA, PGN, HA and TNFα caused increased accumulation of pro-inflammatory cytokines in THP-1 cells (Fig. 6.5A-D). From this initial treatment panel, LPS, LTA, PGN, FLA and TNFα were taken forward as treatments for subsequent experiments.

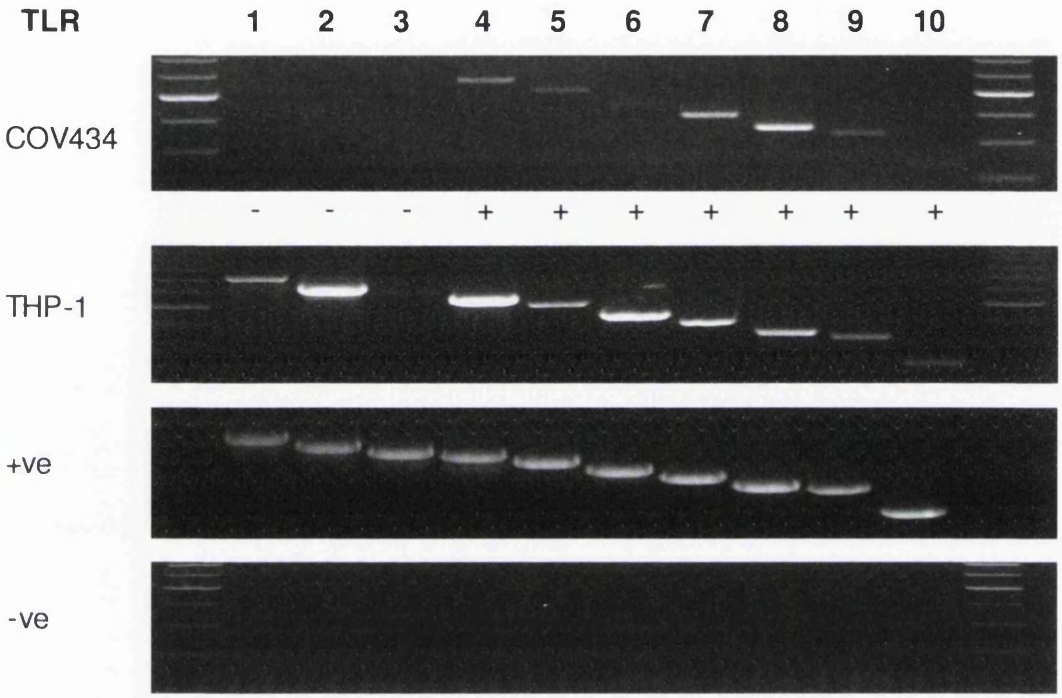


Figure 6.2 COV434 cells expressed *TLR4-10*. Photographs of representative electrophoresis gels of qPCR products visualised using ethidium bromide. Expression of *TLR4-10* was evident in untreated COV434 cells and expression of *TLR1-10* in untreated THP-1 cells. Double-stranded DNA, provided with the Inviogen primers was used as a positive control and water was used as a negative control (-ve). Images are representative of results obtained from RNA extracted on 3 separate occasions. A 100 bp DNA ladder (Promega) is shown on the left and right hand side of each gel.

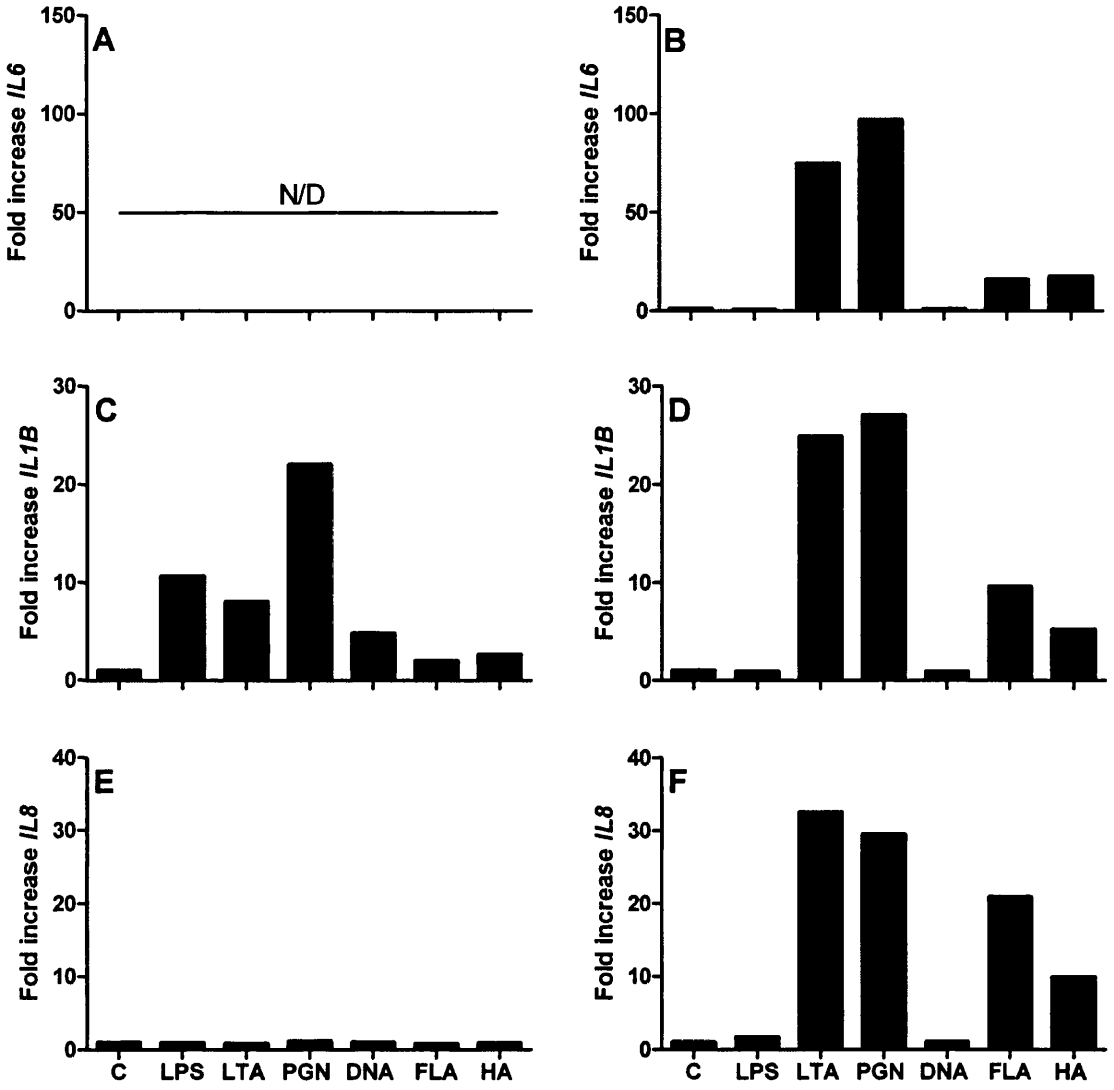


Figure 6.3 mRNA responses by COV434 and THP-1 cells to an initial PAMP panel. COV434 (A-C) and THP-1 cells (D-F) were treated with control medium ('C') or medium containing LPS, LTA, PGN, DNA (all 1 μg/ml), 0.1 μg/ml FLA or 10 μg/ml HA for 3 h. Expression of *IL8* (A, D), *IL6* (B, E) and *IL1B* (C, F) mRNA was measured by real-time PCR. Data is presented as mean of duplicate wells from one initial experiment, relative to control. N/D = below limits of detection.

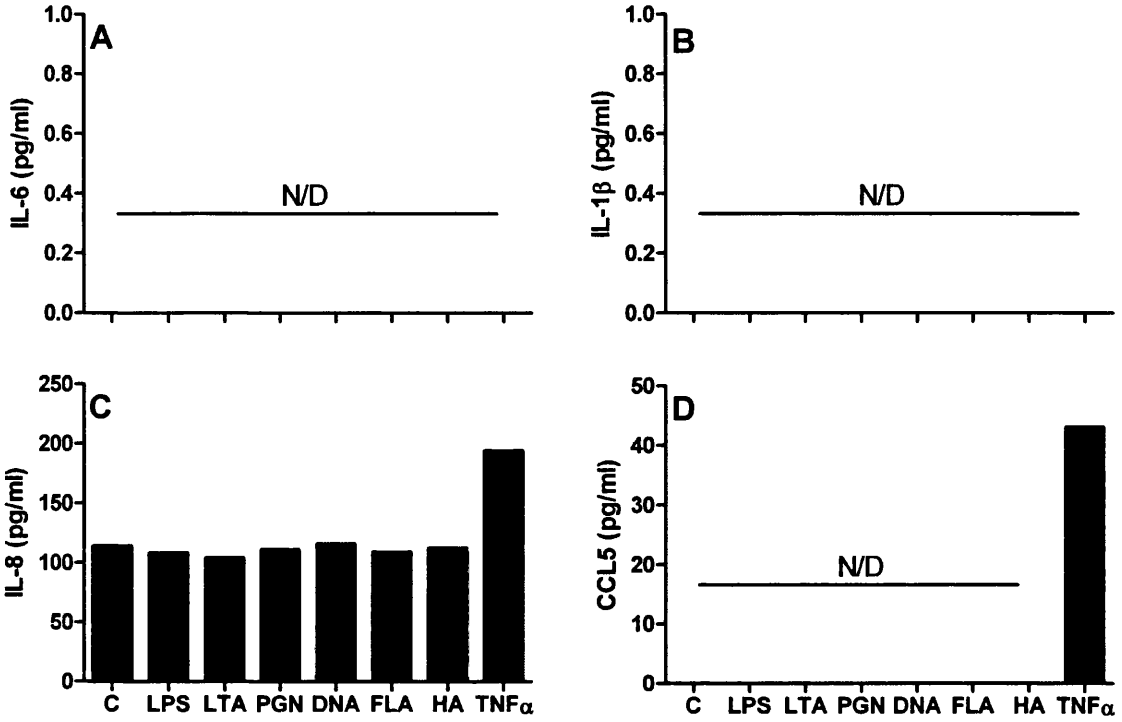


Figure 6.4 Protein responses of COV434 cells to an initial PAMP panel. COV434 cells were treated for 24 h with control medium ('C') or medium containing LPS, LTA, PGN, DNA (all 1 μg/ml), 0.1 μg/ml FLA, 10 μg/ml HA or 20 ng/ml TNFα. Accumulation of IL-6 (A), IL-1β (B), IL-8 (C) and CCL5 (D) was measured in the supernatants by ELISA. Data is presented as mean of duplicate wells from one initial experiment. N/D = below limits of detection.

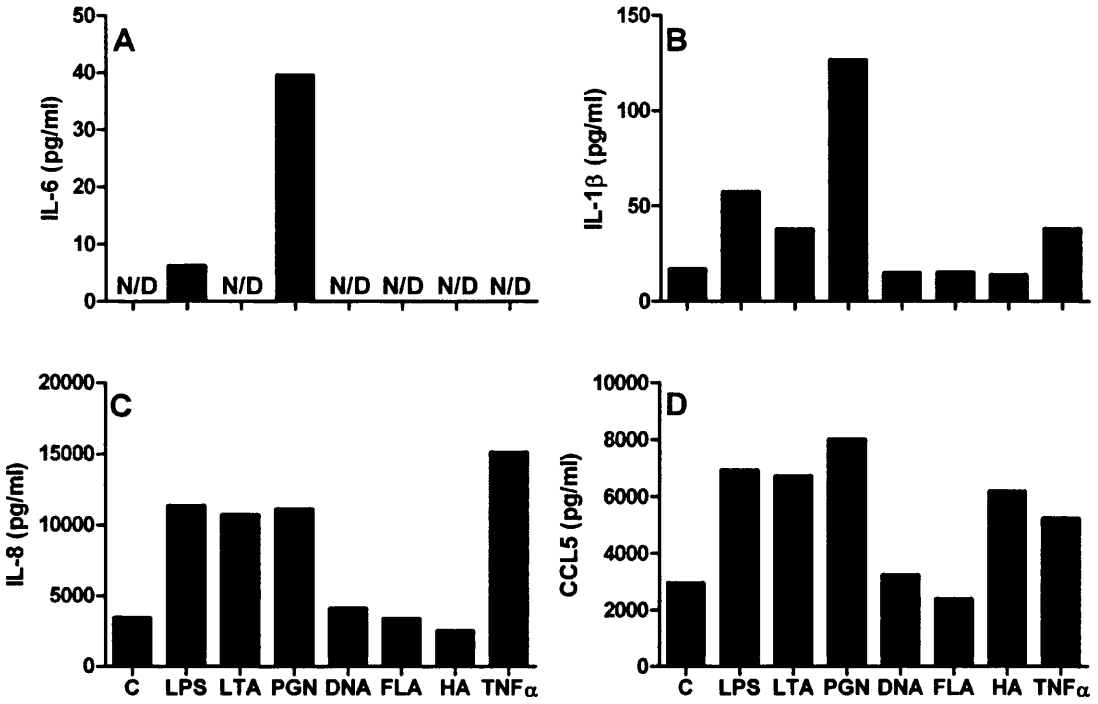


Figure 6.5 Protein responses of THP-1 cells to an initial PAMP panel. THP-1 cells were treated for 24 h with control medium ('C') or medium containing LPS, LTA, PGN, DNA (all 1 μg/ml), 0.1 μg/ml FLA, 10 μg/ml HA or 20 ng/ml TNFα. Accumulation of IL-6 (A), IL-1β (B), IL-8 (C) and CCL5 (D) was measured in the supernatants by ELISA. Data is presented as mean of duplicate wells from one initial experiment.

6.3.5 Response of COV434 cells to PAMPs

In an initial experiment, none of the treatments had an effect on cell viability, cytotoxicity or apoptosis as measured using the ApoTox Glo Triplex Assay (Fig. 6.6A-C). In subsequent experiments, an MTT test was used to assess cell viability; PAMP treatment did not significantly affect cell viability (Fig. 6.7D; $P \geq 0.2$). Treatment of COV434 cells with PAMPs for 3 h significantly increased *IL1B* expression (all treatments $P < 0.05$; Fig. 6.7A), but expression of *IL8* was highly variable (Fig. 6.7B). COV434 cells did not express detectable *IL6* mRNA. Treatment of COV434 cells with PAMPs for 24 h did not significantly increase IL-8 accumulation, although treatment with TNFα increased IL-8 production (Fig. 6.7C; $P < 0.001$). None of the treatments increased the accumulation of IL-1β, IL-6 or CCL5 above the limits of detection of the assay, except that treatment with TNFα increased CCL5 accumulation (control < 2 pg/ml; all PAMPs < 2 pg/ml; TNFα 40 ± 6 pg/ml; $P < 0.05$).

6.3.6 Response of THP-1 cells to PAMPs

Treatment of THP-1 cells with LTA or PGN for 3 h increased expression of *IL6, IL1B* and *IL8* mRNA (Fig 6.8A-C). Treatment of THP-1 cells for 24 h with LPS, LTA, PGN or TNFα increased the accumulation of IL-6, IL-1β, IL-8 and CCL5 in cell supernatants (Fig. 6.9A-D; $P < 0.05$). Although the accumulation of these cytokines and chemokines following treatment with FLA was not significantly greater than control, 3 h treatment with FLA increased *IL1B* mRNA expression 9.86 ± 2.66 fold compared with control (Fig. 6.8B; $P < 0.05$).

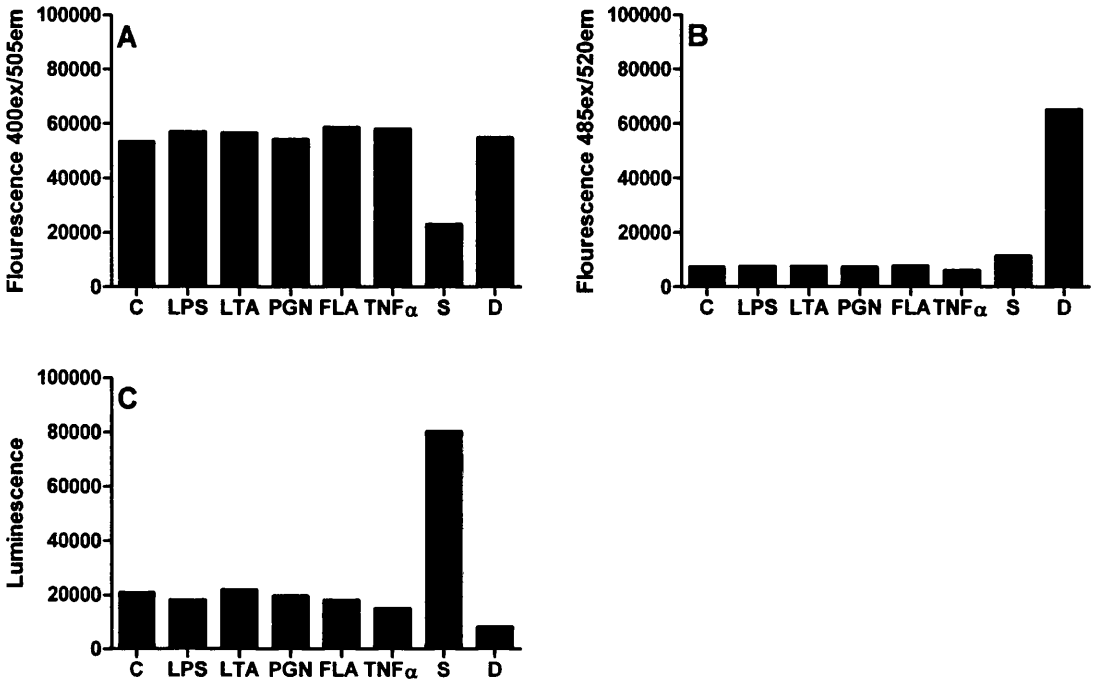


Figure 6.6 Treatment of COV434 cells with PAMPs for 24 h had no significant effect on viability, cytotoxicity or apoptosis. COV434 cells were treated with control medium ('C') or medium containing LPS, LTA, PGN, DNA (all 1 μg/ml), 0.1 μg/ml FLA or 20 ng/ml TNFα for 24 h. Staurosporine (10 μM for 4 h; 'S') and digitonin (30 μg/ml for 15 min; 'D') were used as positive controls. The ApoTox-Glo Triplex assay (Promega, Madison, USA) was used to evaluate the effect of treatments on cell viability (A), cytotoxicity (B) and apoptosis (C). Data presented as mean of duplicate wells from one experiment.

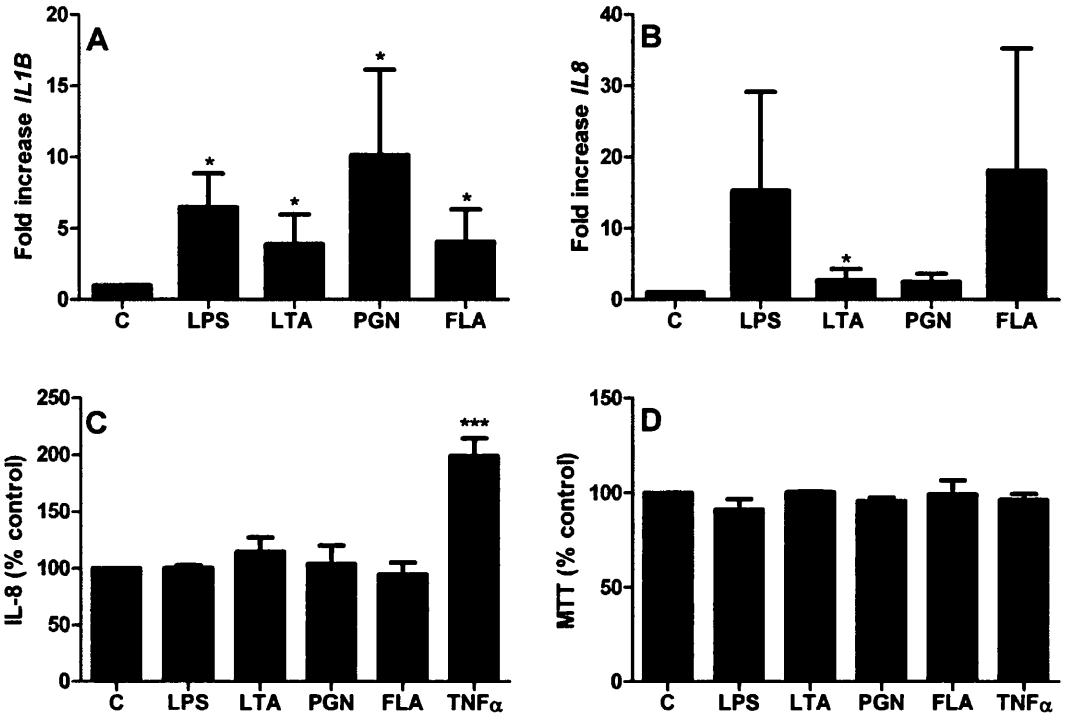


Figure 6.7 COV434 cells responded to PAMPs at the RNA level but did not accumulate protein. COV434 cells were treated for 3 h with control medium ('C') or medium containing PAMPs (LPS, LTA, PGN all 1 μg/ml; FLA 0.1 μg/ml) and the expression of *IL1B* (A) and *IL8* (B) mRNA determined by qPCR (normalised to *ACTB*). Data are presented as mean + SEM for fold change relative to control of 3 independent experiments. Data were analysed using the Mann-Whitney U test; values differ from the control, *P < 0.05. COV434 cells were treated for 24 h with control medium ('C') or medium containing PAMPs (LPS, LTA, PGN all 1 μg/ml; FLA 0.1 μg/ml) or 20 ng/ml TNFα and accumulation of IL-8 (C) measured in the supernatant. The number of cells at the end of 24 h treatment was evaluated by MTT assay (D). Data are presented as mean + SEM, expressed as a percentage of control IL-8 accumulation (C) or control absorbance (D) and represent 4 independent experiments. Data were analysed using ANOVA, with Dunnett's pairwise multiple comparison t-test; values differ from control, *** P < 0.001.

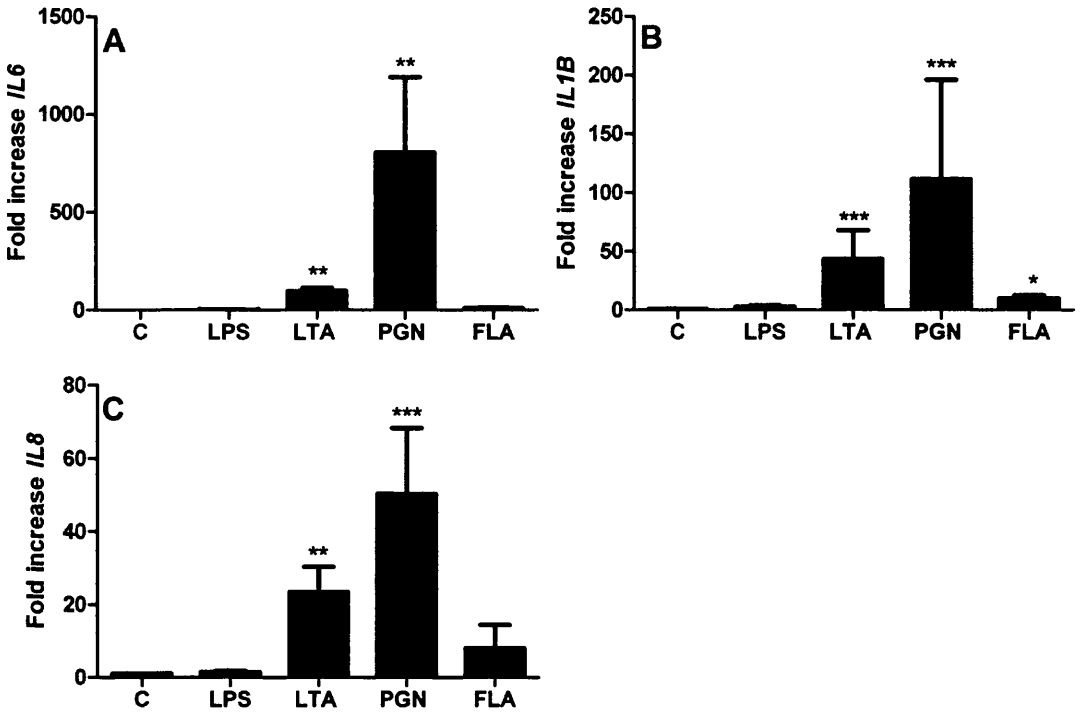


Figure 6.8 THP-1 cells increased expression of mRNA encoding pro-inflammatory cytokines in response to PAMPs. THP-1 cells were treated for 3 h with control medium ('C') or medium containing LPS, LTA, PGN (all 1 μg/ml) or 0.1 μg/ml FLA. RNA was extracted and expression of IL-8, IL-6 and IL-1β (E-G) mRNA measured by qPCR. Data presented as mean + SEM for fold change relative to control from 3 independent experiments. Values differ from control using ANOVA, with Dunnett's pairwise multiple comparison t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

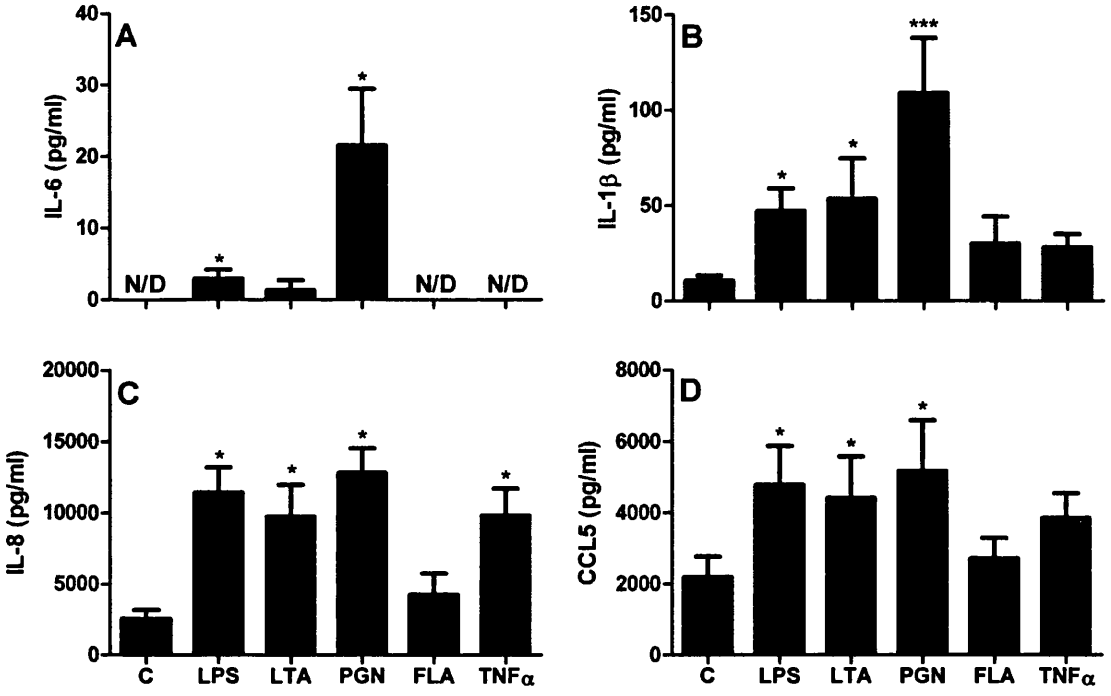


Figure 6.9 THP-1 cells increased accumulation of pro-inflammatory cytokines in response to PAMPs. THP-1 cells were treated for 24 h with control medium ('C') or medium containing LPS, LTA, PGN (all 1 μg/ml), 0.1 μg/ml FLA or 20 ng/ml TNFα and accumulation of IL-8, IL-6, IL-1β and CCL5 (A-D) measured in the supernatants by ELISA. Data are presented as mean + SEM and represent 4 independent experiments. Data were analysed using the Mann-Whitney U test (IL-8, IL-6, CCL5) or ANOVA, with Dunnett's pairwise multiple comparison t-test (IL-1β); values differ from control, * $P < 0.05$, *** $P < 0.001$. N/D = below limits of detection.

6.3.7 Effect of FSH on the response of COV434 cells to PAMPs

The effect of FSH on COV434 cells was explored because FSH modulates TLR4 in murine cells (Shimada et al., 2006). There was no effect of FSH treatment on IL-8 accumulation by COV434 cells in control medium (Fig. 6.10A; $P > 0.8$). Treatment of COV434 cells with 1 ng/ml or 100 ng/ml FSH at the time of PAMP or TNFα treatment, or with 100 ng/ml FSH for 24 h before and during PAMP or TNFα treatment, did not significantly increase the accumulation of IL-8 (Fig. 6.10B-D; all $P \geq 0.6$). As expected, when COV434 cells were treated for 0 - 48 h, there was a significant effect of treatment on the accumulation of oestradiol (Fig. 6.11A; $P < 0.001$). There was no effect of PAMP treatment on oestradiol accumulation when COV434 cells were treated for 24 h with LPS, PGN or TNFα in addition to 100 ng/ml FSH and 2.9 μg/ml androstenedione (Fig. 6.11B; all $P \geq 0.5$).

6.3.8 Using chemical inhibitors decreased the constitutive accumulation of IL-8

The ApoTox-Glo kit was used to determine the most appropriate dose for each inhibitor: a concentration that the COV434 cells could tolerate without a decrease in cell viability or an increase in cytotoxicity or apoptosis, whilst also causing a decrease in IL-8 (Fig. 6.12). The following concentrations were used in further experiments, and were similar to those used in the literature: 4 μM NFκB inhibitor (Abke et al., 2006), 10 μM p38 MAPK inhibitor (Meini et al., 2011), 50 μM JNK inhibitor (Schiavone et al., 2009), 10 μM ERK1/2 inhibitor, 1 μM MEK inhibitor (Scherle et al., 1998). After 24 h treatment of COV434 cells with inhibitors targeting NFκB, p38 MAPK or JNK, there was a significant decrease in IL-8 accumulation ($P < 0.05$; Fig. 6.13A), but inhibiting NFκB or JNK also decreased cell number, as measured by MTT (both $P < 0.05$; Fig 6.13B). After adjusting for cell viability, the inhibitors targeting p38 MAPK, JNK or MEK caused a significant decrease in IL-8 accumulation (all $P = 0.05$).

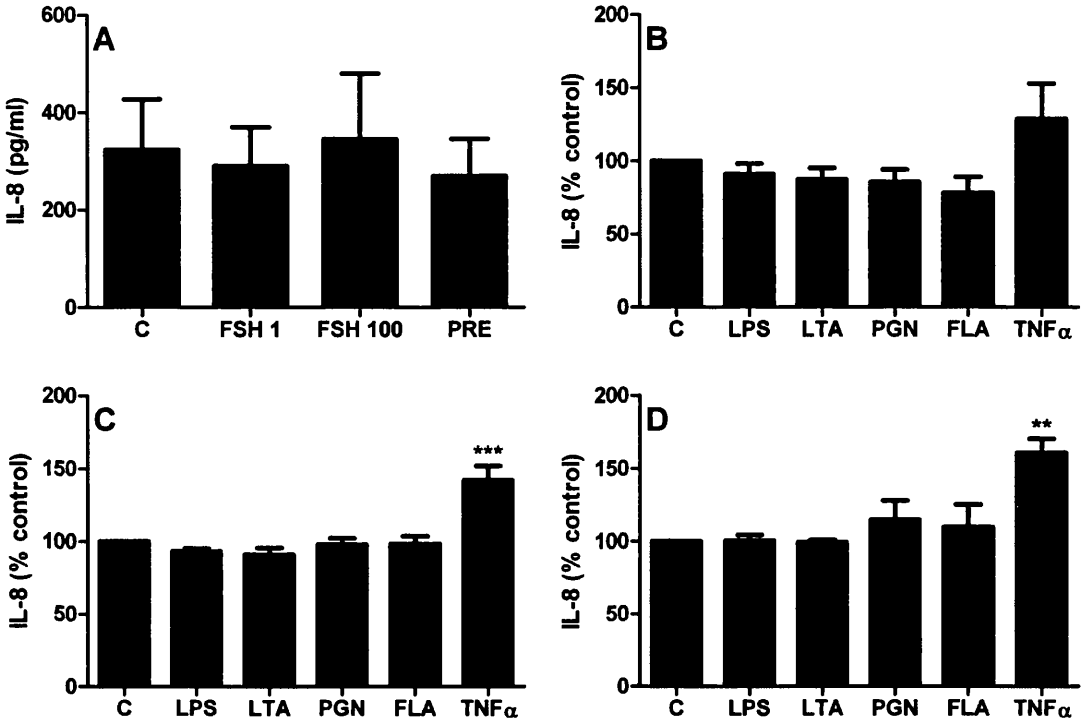


Figure 6.10 FSH did not affect the responsiveness of COV434 cells to PAMPs. (A) COV434 cells were treated for 24 h with control medium ('C') or medium containing 1 ng/ml FSH (FSH 1), 100 ng/ml FSH (FSH 100) or 100 ng/ml FSH after 24 h pre-treatment with 100 ng/ml FSH ('PRE'). Supernatants were collected and the accumulation of IL-8 measured by ELISA. Values are presented as mean + SEM of 3-4 independent experiments and do not significantly differ from control by ANOVA. COV434 cells were treated for 24 h with control medium ('C') or medium containing LPS, LTA, PGN (all 1 μg/ml), 0.1 μg/ml FLA or 20 ng/ml TNFα in the presence of 1 ng/ml FSH (B), or 100 ng/ml FSH (C), or 100 ng/ml FSH after 24 h pre-treatment with 100 ng/ml FSH (FSH pre-treatment) (D). Values are presented as mean + SEM of 3-4 independent experiments relative to control treatment. Values differ from control by ANOVA, using the Dunnett's pairwise multiple comparison t-test to compare treatments with control, ** $P < 0.01$, *** $P < 0.001$.

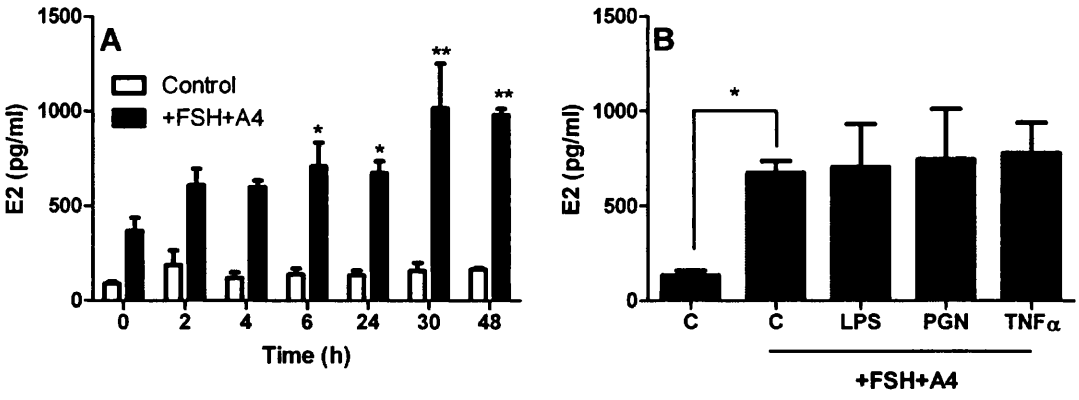


Figure 6.11 COV434 cells produced oestradiol when stimulated with FSH and A4 but this was not perturbed by PAMPs. (A) COV434 cells were treated for 0 - 48 h with control medium or medium containing 100 ng/ml FSH and 2.9 μg/ml A4. Data are presented as mean + SEM of 3 independent experiments, and analysed by ANOVA, using Dunnett's pairwise multiple comparison T-test to compare each time point with time 0, within treatments. (B) COV434 cells were treated for 24 h with control medium ('C') or medium containing 100 ng/ml FSH and 2.9 μg/ml androstenedione (A4) and 1 μg/ml ultrapure LPS, 1 μg/ml PGN or 20 ng/ml TNFα. Data are presented as mean + SEM of 3 independent experiments and analysed using the Mann-Whitney U test, * $P \leq 0.05$, ** $P \leq 0.01$

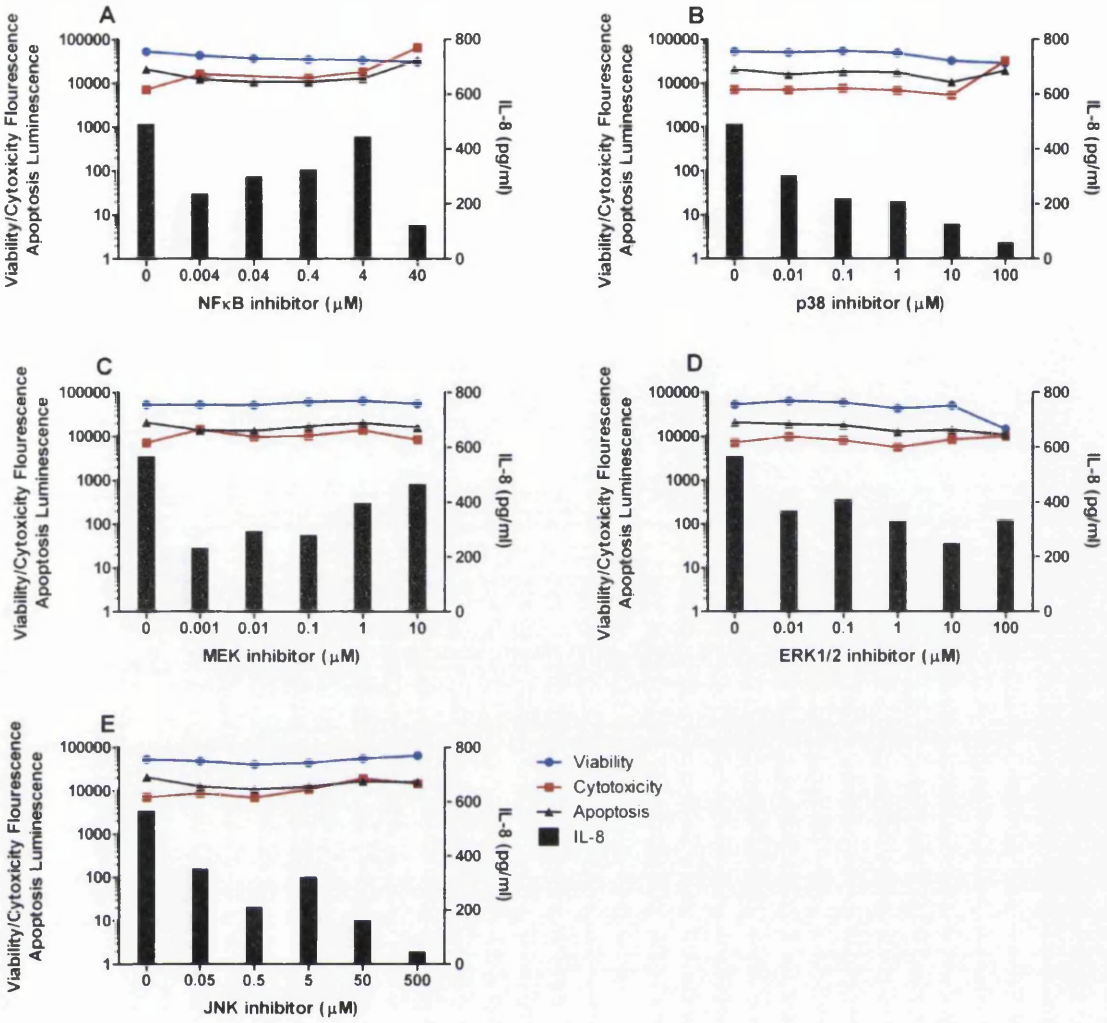


Figure 6.12 Impact of chemical inhibitors on COV434 cells. COV434 cells were treated for 24 h with inhibitors of NFκB (A; 0.004 to 40 μM), p38 MAPK (B; 0.01 to 100 μM), MEK (C; 0.001 to 10 μM), ERK1/2 (D; 0.01 to 100 μM) or JNK (E; 0.05 to 500 μM). Cell viability (blue line), cytotoxicity (red line) and apoptosis (black line) was assessed using the ApoTox Glo Triplex Assay (Promega) and supernatants were collected and the accumulation of IL-8 measured by ELISA (black bars). Data are presented as mean from duplicate wells of 1 experiment.

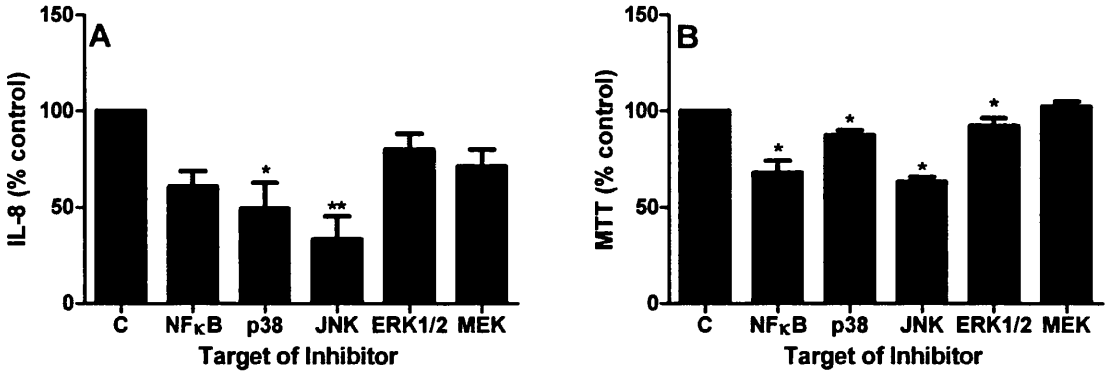


Figure 6.13 Chemical inhibitors of the MAPK pathway decreased the constitutive production of IL-8 by COV434 cells. COV434 cells were treated for 24 h with control medium ('C') or medium containing inhibitors targeting NFκB (4 μM), p38 MAPK (10 μM), JNK (50 μM), ERK1/2 (10 μM) or MEK (1 μM). Supernatants were collected and the concentration of IL-8 measured by ELISA (A). The number of cells at the end of 24 h treatment was evaluated by MTT assay (B). Data are presented as mean + SEM of 3 independent experiments, relative to control and were analysed by ANOVA, using Dunnett's pairwise multiple comparison t-test to compare treatments with control, * $P < 0.05$, ** $P < 0.01$.

6.4 Discussion

The present study tested the hypothesis that the human granulosa cell line COV434 expresses TLRs and can mount an inflammatory response to pathogen associated molecules. The COV434 cells were selected because they have physiological responses similar to primary GCs (Zhang et al., 2000). Primary GCs would be interesting to study but obtaining pure populations of cells without immune cell contamination that may affect the study was not practical (Fedorcsák et al., 2007). The COV434 cells expressed *TLR4-10* and functional responses to PAMPs were evident at the mRNA level. However, treatment of COV434 cells with PAMPs did not produce inflammatory responses evident at the protein level, although PAMPs were effective with a THP-1 monocytic cell line. Of note, the COV434 cells produced IL-8 constitutively, probably via MAPK activation.

The presence of mRNA encoding TLRs in COV434 cells was in agreement with expression of receptors in ovarian GCs from hens (Woods et al., 2009), cattle (Herath et al., 2007) and mice (Shimada et al., 2006). The expression of *TLR4* by COV434 cells agrees with a recent report using the same cells (Woods et al., 2011), which was published after these experiments were completed. The absence of *TLR1-3* in our COV434 cells was interesting: the genes encoding for these TLRs are on chromosome 4 with *TLR2* and *TLR3* close together (4q32 and 4q35 respectively; HUGO), although *TLR1* and *TLR4* are both on 4p14. The published karyotype for COV434 cells shows structural rearrangement only of 22q and trisomy 5 (van den Berg-Bakker et al., 1993), so any mutation is likely to be small scale (at least for *TLR1*), and may possibly just be in the region where the PCR primers bind, rather than a deletion of the whole gene. The lack of expression of *TLR2* found in the present COV434 cell work with commercial primers is in contrast to recently published work showing expression of *TLR2* (Woods et al., 2011), although the latter study used 40 rather than the present study's 35 cycles for PCR.

The present study examined two prototypical cytokines (IL-6 and IL-1β) and two prototypical chemokines (IL-8 and CCL5). These cytokines are involved in a variety of immune processes, including neutrophil, T-cell and leukocyte chemoattraction (CCL5 and IL-8) and the acute-phase protein response (IL-6). However, cytokines also play a role in ovarian physiology and pathology.

Cumulus-oocyte-complex expansion and improved oocyte competence can be induced by IL-6 (Liu et al., 2009). Interleukin 1β decreases progesterone production by FSH-treated GCs (Santana et al., 1996) and may be involved in follicle rupture (Adashi, 1997). CCL5 causes chemotaxis of sperm *in vitro* (Isobe et al., 2002), and may be involved in eosinophil recruitment during granulosa cell luteinisation (Aust et al., 2000). The physiological roles of IL-8 include follicle development and ovulation (Goto et al., 1997, Ujioka et al., 1998, Goto et al., 2002). Cytokines and chemokines are also involved in ovarian pathologies, including cancer, premature ovarian failure, endometriosis, salpingitis and endometritis (Kitaya and Yamada, 2011). For example, high levels of *IL8* expression correlate with the severity of ovarian carcinoma, and blocking *IL8* using siRNA decreases tumour growth (Merritt et al., 2008).

Treatment of COV434 cells with PAMPs for 3 h increased expression of *IL1B* compared with control. The significant increases in *IL1B* expression in COV434 cells treated with PGN probably reflects the ability of PGN to signal through NLR pathways as well as TLR2 (Inohara et al., 2003, Elinav et al., 2011). A recent report also indicates that COV434 cells show nuclear translocation of NFκB following treatment with LPS (Woods et al., 2011). Treatment of COV434 cells with PAMPs for 24 h had no significant effect on cytokine or chemokine protein accumulation, although COV434 cells consitutively produced IL-8. The COV434 cells did not express or accumulate IL-6, which is in contrast to the supernatants of cultured GCs from healthy women, which contain IL-6 but not IL-8 (Carlberg et al., 2000), and in rats where LPS stimulates a dose-dependent increase in IL-6 accumulation at 48 h (Gorospe and Spangelo, 1993). The lack of CCL5 from COV434 cells in the present study agrees with a report of only faint expression of CCL5 in COV434 cells (Aust et al., 2000). In bovine GCs, treatment with LPS or TLR2 ligands (PGN, lipoteichoic acid or the synthetic ligand Pam3CSK4) increased accumulation and expression of IL-8 and IL-6 (Bromfield and Sheldon, 2011). The lack of accumulation of IL-1β despite significant increases in *IL1B* expression in COV434 cells treated with PAMPs, is likely to be due to inactivation of the inflammasome. For IL-1β to be released, cells must receive two signals. The first results in synthesis of pro-IL-1β and components of the inflammasome; the second signal leads to activation of the inflammasome and cleavage of pro-IL-1β to

secretable IL-1β (Guarda and So, 2010, Latz, 2010). A recent study showed that internalised bacterial, adenoviral or mammalian host DNA activated the inflammasome (Muruve et al., 2008). In the present study, COV434 cells received a single 'hit' from a PAMP, which was not transfected and thus was not internalised by the cell. It is likely that the inflammasome was not activated and thus IL-1β was not secreted. On the other hand, THP-1 cells did release mature IL-1β because they were first stimulated with PMA, which causes production of endogenous ATP, providing the second signal to activate the inflammasome (Netea et al., 2009). It is possible that the lack of response to PAMPs is due to the upregulation of nuclear receptor related 1 protein (*NURR1)* in COV434 cells (Alexiadis et al., 2011), because NURR1 protects from LPS-induced inflammation in dopamingeric neurons (Saijo et al., 2009) and macrophages (Bonta et al., 2006).

The COV434 cells express functional FSH receptors, and produce oestradiol in response to FSH and androstenedione treatment (Zhang et al., 2000). Treatment with FSH increases the accumulation of IL-6 in murine GCs (Gorospe and Spangelo, 1993) and FSH can induce *Tlr4* mRNA in mice (Shimada et al., 2006). Treatment of FSH and androstenedione treated bovine GCs with LPS (Herath et al., 2007) or TNFα (Williams et al., 2008) inhibits oestradiol production. However, in the present study, treatment with FSH had no effect on the responsiveness of COV434 cells to PAMPs, and PAMPs or TNFα did not impact oestradiol accumulation. The present study treated COV434 cells with either 1 ng/ml or 100 ng/ml FSH for 24 h. These doses were chosen because 1 ng/ml has been shown to be optimum for granulosa cell culture (Gutiérrez et al., 1997) and 100 ng/ml is the concentration previously used in COV434 cells (Zhang et al., 2000). In the previously mentioned studies, FSH was used at concentrations between 30 and 2000 ng/ml for 48 h (Gorospe and Spangelo, 1993) or 100 ng/ml for 16 h (Shimada et al., 2006). It is not possible to directly compare this literarture with the present study because COV434 cells do not express or produce IL-6. However, it suggests that the difference in response is either due to the difference between using a cell line and using primary cells or a species difference, and not a difference in FSH treatment.

Accumulation of IL-8 in the supernatants of cultured COV434 cells in control medium decreased with increased passage number. This phenonomem has also been reported in BEAS-2B bronchial epithelial cell line (Sanders et al., 1998). It is possible that IL-8 is produced by cells at early passage number in response to the temperature change involved in defrosting stored cell-lines and that the IL-8 accumulation decreases during normal culture. If this is the case, it does not change the findings of this study, as COV434 cells did not respond to PAMPs at any passage number. Accumulation of IL-8 by untreated COV434 cells suggested that COV434 cells produce IL-8 constitutively. This is a common feature of tumour cells, and has been reported in melanoma cells (Schadendorf et al., 1993), tumour-associated brain endothelial cells (Charalambous et al., 2005) and pancreatic carcinoma cells (Hidaka et al., 2002). Treatment with inhibitors of p38 MAPK, JNK and MEK reduced the accumulation of IL-8, even when taking the effects of the chemical inhibitors on cell survival into account. Of note, these signalling molecules are all upstream of the transcription factor activator protein 1 (AP-1), which induces IL-8 mRNA and protein (Wolf et al., 2001, Hoffmann et al., 2002, Kim et al., 2006, Khalaf et al., 2010). Interestingly, AP-1 is constitutively active in COV434 cells (Chu et al., 2004). The COV434 cell line is thought to be developed from a juvenile granulosa cell tumor (Pisarska et al., 2011). Thirty per cent of JGCT have a mutated *gsp* oncogene, leading to constitutive activation of the G_s alpha subunit (Kalfa et al., 2009). This leads to increased cyclic AMP (Buchfelder et al., 1999), which in turn can cause increased activation of AP-1 (Kontny et al., 1992). This mutation has not been reported in COV434 cells, but is a possible mechanism for the constitutive activation of AP-1 and accumulation of IL-8. Other possible mechanisms include the Mothers against decapentaplegic homolog (SMAD) 2/3 pathway, which is associated with *Fosb,* part of AP-1 (Fei et al., 2010). Supporting evidence for this mechanism includes: activity of the SMAD2/3 pathway in JGCT (Middlebrook et al., 2009); activity of SMAD2 in COV434 cells (Stenvers et al., 2009), and the regulation of gonadal tumorogenesis by SMAD3 (Geisel et al., 2007). Inhibiting the SMAD2 pathway or identifying the presence of of the *gsp* oncogene could clarify the role of these pathways in the constitutive activation of AP-1.

In conclusion, the COV434 cell line expressed mRNA for TLR4-10 and responded to PAMPs at the RNA level, but were unable to mount a protein inflammatory response. Although the COV434 cells produced oestradiol, production of this steroid was not perturbed by PAMP treatment. The COV434 cell line constitutively produced IL-8 but did not produce IL-6 in contrast to observations in primary human GCs. So the COV434 human granulosa cell line may be less useful for investigating immunity than for studies of physiology or cancer.

Chapter 7

General Discussion

Bacterial contamination of the bovine uterus is ubiquitous after parturition and is associated with infertility and perturbed ovarian function. Previously, it was reported that LPS from *E. coli* collects in the follicular fluid of animals with uterine disease, and that the GCs lining ovarian follicles express the molecular machinery necessary to sense this LPS. The present thesis examined the response of cells from within ovarian follicles to bacterial PAMPs. It was reported that GCs from emerged and dominant follicles responded to PAMPs but, although exposure to PAMPs during maturation affected the oocyte, the developing embryo seems to be somewhat protected. We also examined the response of a human granulosa cell line to bacterial PAMPs, but discovered many limitations in using a cancerous cell line as a model for investigating the innate immune function of GCs. Whilst the impact on cattle is clear, the effect of PAMPs in humans remains an open question.

Previous investigations have tended to focus on the responses of GCs from the growing pool of emerged follicles to LPS (Bromfield and Sheldon, 2011). However, *in vivo,* perturbation of ovarian function is only detectable when follicles reach dominance, with decreased follicle growth rate and delayed ovulation (Sheldon et al., 2002, Williams et al., 2007). It is possible that only dominant follicles are perturbed by bacterial contamination of the uterus. An alternative, and more likely, hypothesis is that bacterial infections perturb ovarian function at all stages of follicle growth, but that these effects are only detectable in the whole animal once follicles grow larger. The results published in the present thesis support this latter hypothesis, with GCs from both emerged and dominant follicles responding to the bacterial PAMPs, LPS and PAM. Although there were many similarities between the responses of GCs from emerged and dominant follicles, some differences also emerged (Table 7.1). Granulosa cells from both cell populations expressed all 10 TLRs, although the expression pattern of these receptors differed between emerged and dominant follicles. It was particularly interesting that both cell populations expressed a high level of *TLR3,* thus the response of GCs to viruses or viral PAMPs, whilst beyond the scope of this project, would be interesting to investigate. In particular, the effect of bovine herpesviruses and bovine diarrhoea virus would be interesting to study, as these infections are also associated with ovarian dysfunction and endometritis or mastitis (Grooms et al., 1998, Donofrio et al., 2009).

Table 7.1 The responses of GCs isolated from emerged and dominant follicles to PAMPs.

Emerged	Dominant
Expressed *TLR1-10*; high *TLR3*; low *TLR8*	Expressed *TLR1-10*; high *TLR1, TLR3, TLR5, TLR6*; low *TLR8*
LPS → ↑*TLR2, TLR3, TLR5*; ↓*TLR7* PAM → ↑*TLR2*; ↓*TLR4, TLR6, TLR7, TRL10*	LPS → ↑*TLR2*; ↓*TLR4, TLR6, TLR7, TLR8, TLR10* PAM → ↑*TLR2*; ↓*TLR6, TLR7, TLR8, TLR9*
No priming effect	LPS or PAM ↑IL-6 response to subsequent PAM treatment
LPS or PAM → ↑IL-6, IL-1β, TNF-α, IL-8 FLA → ↑IL-6	LPS or PAM → ↑IL-6, IL-1β, IL-8 FLA → no effect
1 μg/ml LPS → 1696 pg/ml IL-6 1 μg/ml PAM → 1401 pg/ml IL-6 1 μg/ml FLA → 46 pg/ml IL-6	1 μg/ml LPS → 843 pg/ml IL-6 1 μg/ml PAM → 1200 pg/ml IL-6 1 μg/ml FLA → N/D IL-6
LPS, PAM or FLA → ↑*IL6, IL1B, IL10, TNF, IL8, CCL5*	LPS or PAM → ↑*IL6, IL1B, IL10, TNF, IL8, CCL5* FLA → not tested
PAM → ↑*EGFR*	LPS → ↓*EGFR, CYP19A1* PAM → ↓*LHCGR, CYP19A1*
LPS or PAM → ↑p-ERK1/2, p-p38	LPS or PAM → ↑p-ERK1/2, p-p38
E2 → ↓IL-6; EGF → ↑IL-6 EGF+PAM → ↑IL-6 (compared to PAM alone)	EGF or LH → ↑IL6 E2+LPS → ↓IL-6 ; EGF+LPS → ↑IL-6 (compared to LPS alone) E2+PAM → ↓IL-6 ; EGF+PAM → ↑IL-6 (compared to PAM alone)
Inhibiting NFκB, MEK, p38 or MEK+JNK ↓IL-6 LPS response Inhibiting NFκB or p38↓IL-6 PAM response	Inhibiting NFκB, MEK, p38, JNK or p38+MEK ↓IL-6 LPS response Inhibiting NFκB, MEK or p38 ↓IL-6 PAM response
Effect of PAMPs on E2 and P4 not tested	LPS or PAM → ↓E2 LPS → ↓P4

One interesting observation was that GCs isolated from emerged follicles tended to have a greater response to bacterial PAMPs than those isolated from dominant follicles, with increased cytokine production in response to LPS, PAM or FLA. This observation supports the theory that bacterial infections affect emerged follicle health, with the impact only detectable once the follicles reach dominance, in addition to bacterial infections impacting dominant follicle function directly.

Prior to the investigations presented in the current thesis, a working model summarising current knowledge of endocrine and innate immune function within ovarian follicles was described (Fig 1.9). The present work produced observations which develop these basic models, and provides further links between these two ancient systems (Fig 7.1 and 7.2). Of particular note was the interplay between PAMPs, IL-6 and the LH-EGF pathway, especially in GCs isolated from dominant follicles, where EGF is likely to play a greater role in promoting maturation of the oocyte. Treatment of GCs isolated from dominant follicles with LPS or PAM decreased expression of *EGFR* and *LHCGR*, respectively. Supposing that these reductions in mRNA translate to reduced protein, these reductions in receptor expression may delay maturation of the oocyte. Potentially, this is a protective mechanism against ovulation occurring in a 'dirty' environment. Increased LH enhances EGFR activation (Panigone et al., 2008), potentially through increased production of EGF, thus it was unsurprising that treatment of GCs with LH or EGF enhanced constitutive accumulation of IL-6 (potential mechanisms have been previously discussed in chapters 3 and 4). However, it was surprising that treatment of GCs with LH in tandem with LPS or PAM did not enhance PAMP-stimulated IL-6 accumulation, even though EGF co-treatment augmented PAMP-stimulated IL-6 accumulation by around 50%. This suggests that PAMPs may interfere with the LH-stimulated production of EGF. Previously, this pathway was described in mouse and pig as involving cAMP, PKA, p38 and ERK1/2 (Yamashita et al., 2009, Hsieh et al., 2011) and, interestingly, p38 and ERK1/2 were both involved in the response of GCs to LPS and PAM in the present thesis. Thus, it would be of interest for further investigations to focus on the interactions between PAMPs and EGF, in particular, the effect of PAMPs on the accumulation of EGF-like factors.

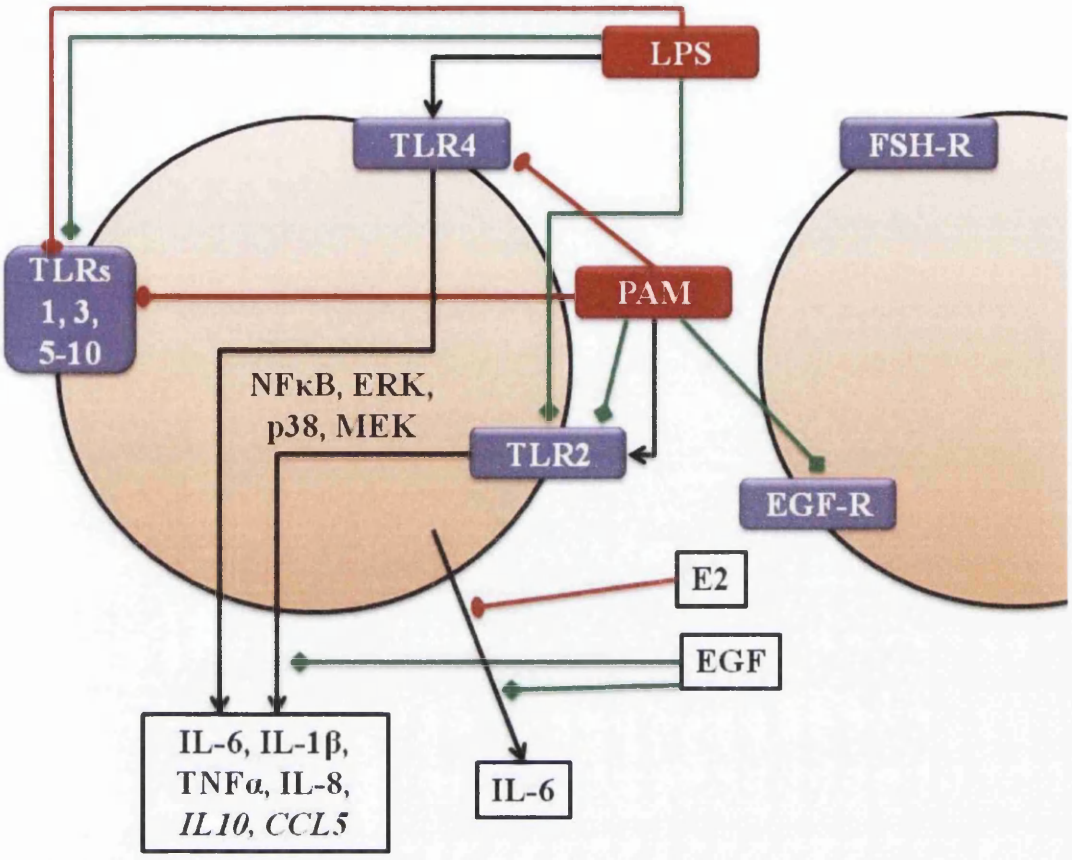


Figure 7.1 A summary of the interactions between innate immune and endocrine pathways in GCs isolated from emerged follicles. Granulosa cells isolated from emerged follicles expressed *FSHR* and *EGFR*. They also expressed all 10 TLRs. PAM and LPS bound TLR2 and TLR4, respectively, inducing an intracellular cascade resulting in increased expression and accumulation of cytokines and chemokines. Granulosa cells also produced IL-6 constitutively. Treatment of GCs with LPS impacted on expression of some TLRs, of particular note increasing expression of *TLR2*. Treatment of GCs with PAM decreased expression of TLRs including *TLR4* and increased expression of *TLR2*. PAM treatment also increased expression of *EGFR*. Oestradiol treatment decreased the constitutive accumulation of IL-6, which was enhanced by EGF treatment. Red lines represent a decrease in production or expression, whereas green lines represent an increase.

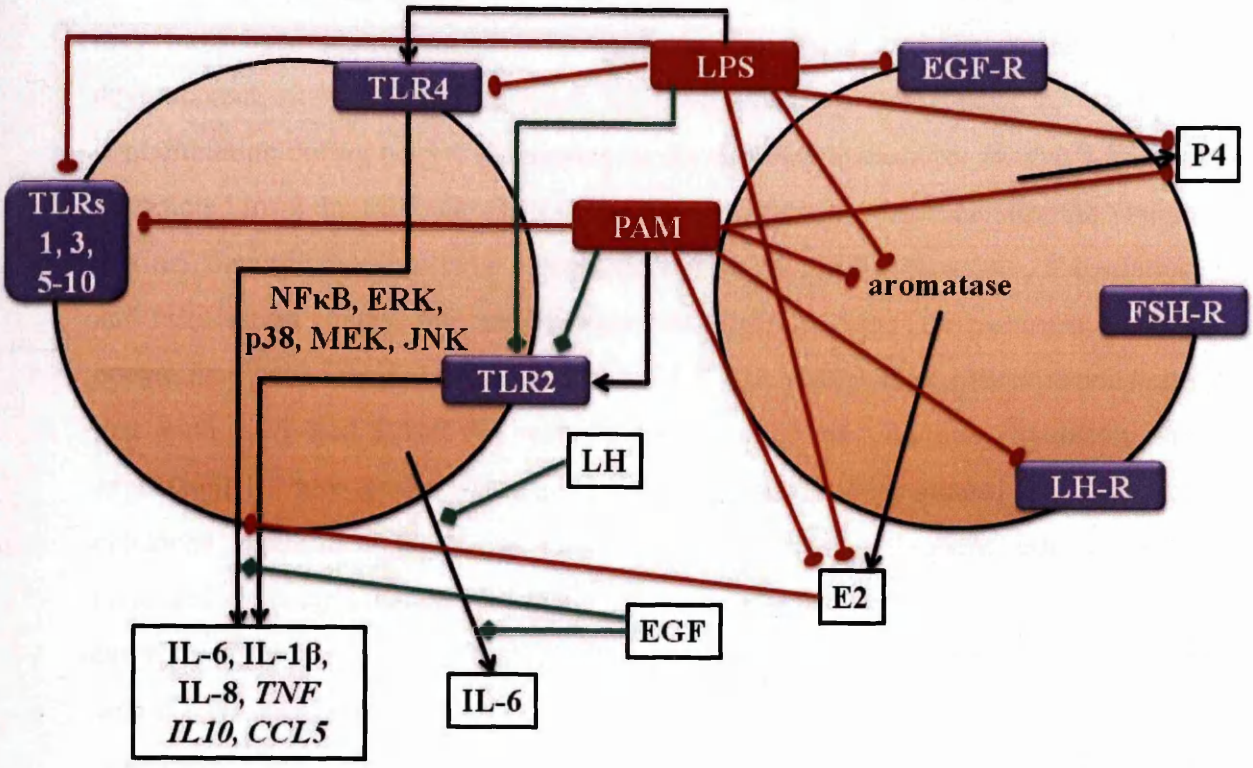


Figure 7.2 A summary of the interactions between innate immune and endocrine pathways in GCs isolated from dominant follicles. Granulosa cells isolated from dominant follicles expressed *FSHR, LHCGR, EGFR* and *CYP19A1.* They also expressed all 10 TLRs. PAM and LPS bound TLR2 and TLR4, respectively, inducing an intracellular cascade resulting in increased expression and accumulation of cytokines and chemokines. Granulosa cells also produced IL-6 constitutively. Treatment of GCs with LPS decreased expression of TLRs, including *TLR4,* and increased expression of *TLR2.* Treatment of GCs with PAM decreased expression of some *TLR*s and increased expression of *TLR2.* PAM treatment also decreased expression of *LHCGR*, whereas LPS treatment decreased expression of *EGFR.* Treatment with LPS or PAM decreased expression of *CYP19A1* and accumulation of oestradiol or progesterone. Oestradiol treatment decreased the LPS- or PAM-stimulated accumulation of IL-6, which was enhanced by EGF treatment. Treatment of GCs with LH or EGF increased constitutive IL-6 accumulation. Red lines represent a decrease in production or expression, whereas green lines represent an increase.

Arguably the most novel aspect of this research was the impact of bacterial contamination during oocyte maturation on subsequent embryo health. Although it may have been interesting to investigate the impact of PAMPs during embryo development, it was more relevant to concentrate on the effects of bacterial contamination during oocyte maturation on the subsequent embryo. *In vivo,* LPS can be isolated from the follicular fluid of previously infected cows even after the visible uterine contamination has been cleared (Herath et al., 2007). Therefore, if ovulation and fertilisation occurs, the embryo may develop in a 'clean' environment, yet the oocyte may have matured whilst bathed in LPS. The results in chapter 5 demonstrate that both LPS and PAM not only impact directly on cumulus expansion and expression of key genes involved in early embryo development, but that this enhanced maturation translates into changes in the subsequent embryo with increased cleavage rates in parthenogenetically activated oocytes. It seems that this early maturation continues through into early first and subsequent cleavages. It would have been fascinating to continue development of these parthenotes into the blastocyst stage. However, this was not possible due to time and technical limitations. There was little impact of LPS or PAM exposure during IVM on parthenote gene expression after 42 h culture, thus it would have been interesting to discover whether the accelerated growth continued through early embryogenesis, and whether it led to increased rates of embryo death. It would be interesting to extend these *in vitro* studies into the live animal, maturing bovine oocytes *in vitro* in the presence of PAMPs and then returning to the cow, to test whether there is any impact on implantation or embryo health.

The work presented in this thesis highlights the importance of using a valid model for investigations. Primary bovine GCs responded to LPS and PAM and constitutively produced few inflammatory mediators. Although the model was not perfect, in that the culture of the GCs without serum was not possible, the GCs continued to function physiologically, producing oestradiol in response to FSH and COCs expanding in response to gonadotrophin stimulus. In contrast, the COV434 cells constitutively produced high concentrations of IL-8 and did not respond at the protein level to PAMPs. Although cell-lines have a purpose and are often offer the best option for human work, it is important to recognise the difficulties with using abnormal cancerous cells.

One important question remaining to be answered is how LPS collects inside the follicle. It seems likely that endotoxin from invading *E. coli* in the uterus passes into the local vasculature which then runs to the ovary. Perhaps the LPS simply diffuses into the follicular fluid, but then why does it not diffuse out again once the concentration of LPS in the blood is lower than in the follicular fluid? The blood-follicle barrier was previously estimated to be 100 kDa (Clarke et al., 2006) somewhat larger than the size of LPS monomers of 10 kDa (Petsch and Anspach, 2000). However, LPS also forms large aggregates, up to 1000 kDa in size (Petsch and Anspach, 2000). Maybe LPS aggregates once inside the follicle but does not aggregate in the blood due to circulatory movement and pressure? Another possible explanation is that LPS binds to a component of follicular fluid. In the present work, it was shown that HMW HA dampens the inflammatory effect of LPS. Potentially, HA could bind LPS, thus decreasing the affinity of LPS to TLR4. We know that HA is physically sticky, but perhaps it is also chemically sticky? Investigations could simply start with preparing a mix of LPS and hyaluronan, allowing time for binding to occur, soaking isolated follicles in the soup and then measuring LPS in aspirated follicular fluid, to test whether a greater concentration of LPS is present in follicles soaked in LPS alone rather than LPS+HA. However, it is unlikely that this simple approach *ex vivo* would yield meaningful results. A more complex but physiological method could include the use of knock-out mice to disrupt formation of the follicular fluid and test how LPS accumulated after uterine infusion. This could prove challenging as the *Has2*$^{-/-}$ knockout mouse dies close to birth, thus conditional knockout mice would have to be used, silencing similar genes only in ovarian GCs (Roughley et al., 2011)

The results presented in this thesis, in concert with those published in recent papers (Bromfield and Sheldon, 2011, Bromfield and Sheldon, 2013), have provided greater understanding of the impact of bacterial infections on ovarian health at different stages of follicular development and embryogenesis. It seems that ovarian follicles are sensitive to LPS at the early stages of development, before becoming less sensitive whilst secondary follicles. However, as the antral stage is reached, the follicles are once again perturbed by PAMPs (Fig 7.3). Oocyte maturation is also perturbed by LPS and PAM, but the early embryo seems to be somewhat protected from bacterial infections.

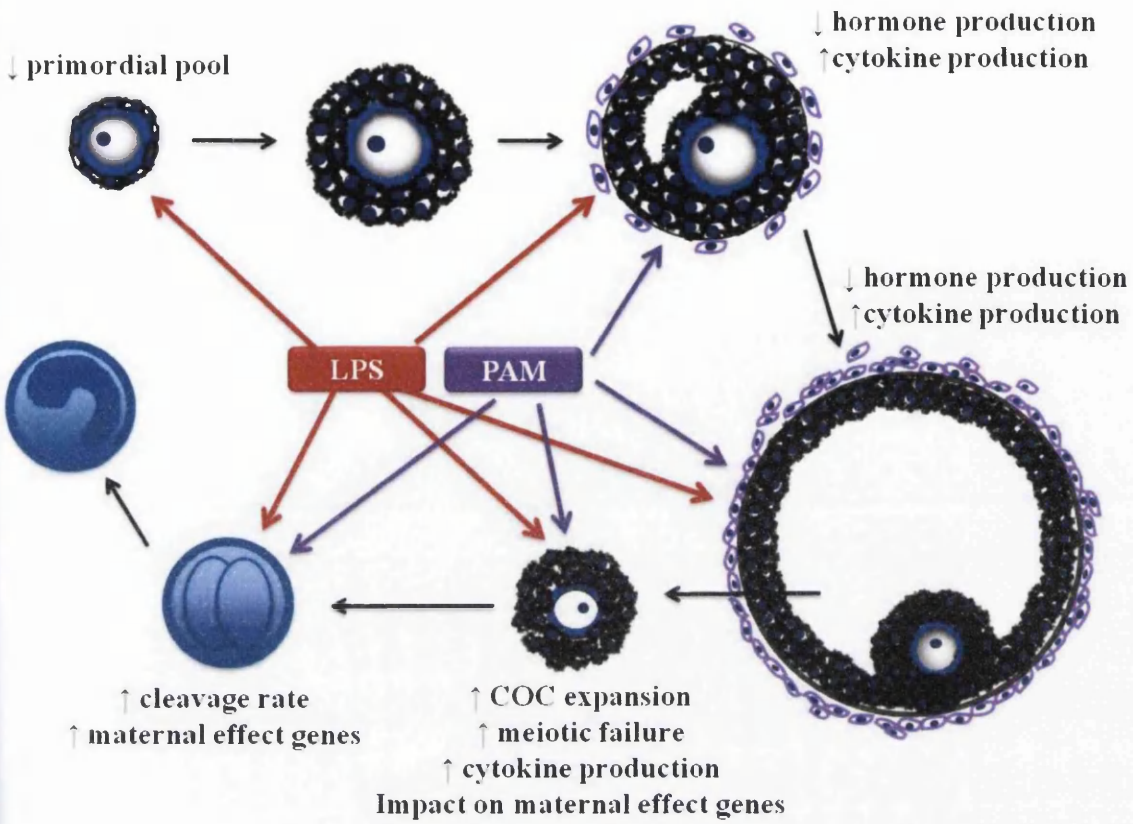


Figure 7.3 The bacterial PAMPs LPS and PAM impact ovarian follicle health at most stages of development. Recent *ex vivo* experiments have shown that LPS decreases the primordial pool of bovine follicles, showing an impact of infection at even this early stage of follicular development, although secondary follicles seem to be somewhat protected from endotoxin exposure (Bromfield and Sheldon, 2013). Once follicles emerge, *in vitro* results show a perturbation of granulosa function with increased cytokine production (chapter 3) and decreased hormone production (Herath et al., 2007). At the follicle grows, perturbation becomes detectable *in vivo* and granulosa cell function is further perturbed (chapter 4). Oocyte maturation in the presence of LPS or PAM increases cumulus expansion, cytokine production, meiotic failure (Bromfield and Sheldon, 2011) and impacts on the expression of maternal effect genes (chapter 5). The early oocyte initially develops earlier after maturation in the presence of PAMPs, but there seems to be little effect on the subsequent embryo (chapter 5).

Advancing our knowledge of how infection impacts fertility could help to address a number of problems humanity faces. From an economic point of view, much of the developed world is in the midst of a period of uncertainty, as developing nations learn the skills the Western World previously exclusively offered, but can provide at lower prices. With bovine uterine disease estimated to cost the EU alone 1.4 billion Euros per year (Sheldon et al., 2009), the potential to reduce the portion of costs attributable to reduced fecundity and decreased milk yield is an attractive proposition for farmers and government alike.

Developing tools to improve fertility is also of economic interest to the pharmaceutical industry. In the previous years, the number of blockbuster drugs launched has decreased whilst the number of patents expiring has increased, resulting in declining profits for many companies and a burgeoning business for generic drugs (Harrison, 2013). The treatment of infertility is an emerging market able to target animal and human health. The current trend for professional women to delay motherhood, combined with increased incidence of bacterial sexually-transmitted infections, such as *Chlamydia*, provides an affluent potential therapeutic group (Vickers and Osgood, 2010).

Alternatively, we can look at infertility from a human standpoint. With the human population ever expanding, our need for fuel is exponentially rising. Cattle provide a means of converting grass, which is indigestible to humans, to milk or beef, providing a source of protein and fat. Thus, by increasing bovine fertility, there is less wastage in the farming system and food supply is increased. However, this is incredibly energy-inefficient and, in the author's opinion, will not solve any problems with food shortage. As a general rule in nature, when the food supply to a species increases, the numbers of that species expand. Humans are unlikely to be an exception. Thus, a greater understanding of the mechanisms underlying fertility could be used to control population expansion, allowing sustainable development. As a therapeutic this could be used as a prophylactic treatment to decrease fertility.

Infections of the female genital tract are common in humans and cattle and impact the environment, global food security, animal welfare and the economy. In addition to impacting the uterus, mammary gland and brain of infected animals, these infections also perturb ovarian function. Cattle with endometritis or mastitis have decreased dominant follicle growth rate and lower peripheral concentrations of oestradiol. The present thesis investigated the role of bacterial pathogen-associated molecular patterns on the GCs and oocytes within bovine ovarian follicles. Previous investigations had focussed on the effect of the prototypical PAMP LPS on GCs isolated from emerged follicles. The present work extended these findings, exploring the impact of the TLR2 agonist PAM and TLR5 agonist FLA on GCs from both emerged and dominant follicles. In addition to further dissecting the inflammatory pathways activated in these cells through the use of siRNA, Western blots and biochemical inhibitors, the crosstalk between endocrine and innate immune pathways was examined, identifying an interesting link between EGF and IL-6. The natural progression of follicle development was followed after dominance, to focus on the impact of bacterial PAMPs in the follicle on the oocyte and subsequent embryo, demonstrating a striking perturbation of early events during oocyte maturation and embryogenesis. Lastly, a human granulosa cell line was investigated. Although these cells were found to express *TLR*s, the abnormality of these cells was demonstrated and will hopefully act as a caution for others using cell-lines to investigate physiology and non-cancerous pathology.

The findings presented in the current thesis provide a greater understanding of ovarian physiology and pathology. With this insight, potential therapeutics to treat ovarian dysfunction and infertility caused by bacterial infection can start to be explored, in addition to the creation of prophylactics which could intentionally limit fertility.

Appendix 1

Dulbecco's Phosphate Buffered Saline Components (Sigma)	Concentration (mg/L)
Potassium Chloride	200
Potassium Phosphate (anhydrous)	200
Sodium Chloride	8000
Disodium Phosphate (anhydrous)	1150

RPMI 1640 Components (Sigma)		Concentration (mg/L)
Amino Acids	Glycine	10
	L-Arginine hydrochloride	200
	L-Asparagine	50
	L-Aspartic acid	20
	L-Cystine	65.2
	L-Glutamic Acid	20
	L-Glutamine	300
	L-Histidine	15
	L-Hydroxyproline	20
	L-Isoleucine	50
	L-Leucine	50
	L-Lysine hydrochloride	40
	L-Methionine	15
	L-Phenylalanine	15
	L-Proline	20
	L-Serine	30
	L-Threonine	20
	L-Tryptophan	5
	L-Tyrosine	28.83
	L-Valine	20
Vitamins	Biotin	0.2
	Choline chloride	3
	D-Calcium pantothenate	0.25
	Folic Acid	1
	Niacinamide	1
	Para-Aminobenzoic Acid	1
	Pyridoxine hydrochloride	1
	Riboflavin	0.2
	Thiamine hydrochloride	1
	Vitamin B12	0.005
	i-Inositol	35
Inorganic Salts	Calcium nitrate	100
	Magnesium Sulfate	48.84
	Potassium Chloride	400
	Sodium Bicarbonate	2000
	Sodium Chloride	6000
	Sodium Phosphate dibasic anhydrous	800
Other	D-Glucose	2000
	Glutathione (reduced)	1
	Phenol Red	5.3

Medium 199 (M199) Components (Gibco)		Concentration (mg/L)
Amino Acids	Glycine	50
	L-Alanine /hydrochloride	25 /70
	L-Aspartic acid	30
	L-Cysteine hydrochloride-H2O	0.1
	L-Cystine 2HCl	26
	L-Glutamic Acid	66.8
	L-Glutamine	100
	L-Histidine hydrochloride-H2O	21.88
	L-Hydroxyproline	10
	L-Isoleucine	40
	L-Leucine	60
	L-Lysine hydrochloride	70
	L-Methionine	15
	L-Phenylalanine	25
	L-Proline	40
	L-Serine	25
	L-Threonine	30
	L-Tryptophan	10
	L-Tyrosine disodium salt dihydrate	58
	L-Valine	25
	Alpha-tocopherol Phosphate	0.01
Vitamins	Ascorbic Acid	0.05
	Biotin	0.01
	Choline chloride	0.5
	D-Calcium pantothenate	0.01
	Folic Acid	0.01
	Menadione (Vitamin K3)	0.01
	Niacinamide	0.025
	Nicotinic acid (Niacin)	0.025
	Para-Aminobenzoic Acid	0.05
	Pyridoxal hydrochloride	0.025
	Pyridoxine hydrochloride	0.025
	Riboflavin	0.01
	Thiamine hydrochloride	0.01
	Vitamin A / D2	0.1 /0.1
	i-Inositol	0.05
	Calcium Chloride	264
	Ferric nitrate	0.7
Inorganic Salts	Magnesium Sulfate	200
	Potassium Chloride	400
	Sodium Bicarbonate	2200
	Sodium Chloride	6800
	Sodium Phosphate monobasic	158
	2-deoxy-D-ribose	0.5
	Adenine sulfate	10
Other	Adenosine 5'-phosphate	0.2
	Adenosine 5'-triphosphate	1
	Cholesterol	0.2
	D-Glucose	1000
	Glutathione	0.05
	Guanine hydrochloride	0.3
	Hypoxanthine	0.3
	Phenol Red	20
	Ribose	0.5
	Sodium acetate-3H2O	83
	Thymine	0.3
	Tween 80	20
	Uracil	0.3
	Xanthine	0.344

Dulbecco's Modified Eagle's Medium Components (Gibco)		Concentration (mg/L)
Amino Acids	Glycine	30
	L-Alanyl-Glutamine	862
	L-Arginine hydrochloride	84
	L-Cystine	48
	L-Histidine hydrochloride-H2O	42
	L-Isoleucine	105
	L-Leucine	105
	L-Lysine hydrochloride	146
	L-Methionine	30
	L-Phenylalanine	66
	L-Serine	42
	L-Threonine	95
	L-Tryptophan	16
	L-Tyrosine	72
	L-Valine	94
Vitamins	Choline chloride	4
	D-Calcium pantothenate	4
	Folic Acid	4
	Niacinamide	4
	Pyridoxine hydrochloride	4
	Riboflavin	0.4
	Thiamine hydrochloride	4
	i-Inositol	7.2
Inorganic Salts	Calcium Chloride	264
	Ferric Nitrate	0.1
	Magnesium Sulfate	200
	Potassium Chloride	400
	Sodium Bicarbonate	3700
	Sodium Chloride	6400
	Sodium Phosphate monobasic	141
Other	D-Glucose (Dextrose)	1000
	Phenol Red	15
	Sodium Pyruvate	110

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