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Follicular expression of pro-inflammatory cytokines tumour necrosis factor-α (TNFα), interleukin 6 (IL6) and their receptors in cattle: TNFα, IL6 and macrophages suppress thecal androgen production *in vitro*

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Abstract

Pro-inflammatory cytokines secreted by macrophages and other cell-types are implicated as intra-ovarian factors affecting different aspects of ovarian function including follicle and corpus luteum 'turnover', steroidogenesis and angiogenesis. Here, we compared granulosal (GC) and thecal (TC) expression of TNF, IL6 and their receptors (TNFRSF1A, TNFRSF1B, IL6R) during bovine antral follicle development; all five mRNA transcripts were detected in both GC and TC and statistically significant cell-type and follicle stage-related differences were evident. Since few studies have examined cytokine actions on TC steroidogenesis, we cultured TC under conditions that retain a non-luteinized 'follicular' phenotype and treated them with TNFα and IL6 under basal and LH-stimulated conditions. Both TNFα and IL6 suppressed androgen secretion concomitantly with CYP17A1 and LHCGR mRNA expression. In addition, TNFα reduced INSL3, HSD3B1 and NOS3 expression but increased NOS2 expression. IL6 also reduced LHCGR and STAR expression but did not affect HSD3B1, INSL3, NOS2 or NOS3 expression. Since macrophages are a prominent source of these cytokines *in vivo* we next co-cultured TC with macrophages and observed an abolition of LH-induced androgen production accompanied by a reduction in CYP17A1, INSL3, LHCGR, STAR, CYP11A1 and HSD3B1 expression. Exposure of TC to bacterial lipopolysaccharide also blocked LH-induced androgen secretion, an effect reduced by a toll-like receptor blocker (TAK242). Collectively, the results support an inhibitory action of macrophages on thecal androgen production, likely mediated by their secretion of pro-inflammatory cytokines that downregulate expression of LHCGR, CYP17A1 and INSL3. Bovine theca interna cells can also detect and respond directly to lipopolysaccharide.

Introduction

Cyclic ovarian function involves serial tissue remodelling associated with follicular growth, atresia, ovulation, and the generation and regression of corpora lutea. After ovulation there is breakdown, repair and orderly regeneration of ovarian tissues. Whilst a resident population of ovarian macrophages exists, during the peri-ovulatory phase, additional macrophages accumulate in the ovary and secret pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα), interleukin-6 (IL6) and interleukin-1b (IL1b) that have local actions on ovarian follicular cells and contribute to the ovulatory process (Cohen, et al. 1999, Turner, et al. 2011, Wu, et al. 2004). Likewise, towards the end of the cycle, macrophages infiltrate the corpus luteum and their inflammatory mediators appear to play a prominent role in luteolysis (Okuda and Sakumoto 2003, Walusimbi and Pate 2013). The cyclic infiltration of macrophages into ovarian tissue as well as their presence elsewhere in the female reproductive tract is strong evidence for their multifaceted role in the female reproductive process (Brannstrom, et al. 1993, Miller and Hunt 1996, Sheldon, et al. 2014, Walusimbi and Pate 2013).

TNFα is a multifunctional pro-inflammatory cytokine belonging to the TNF superfamily. It is mainly secreted by activated macrophages and other immune cells including T lymphocytes although many other cell types including vascular endothelial cells, skeletal muscle cells and ovarian GC have also been shown to express and/or secrete TNFα (Peake, et al. 2015, Price and Sheldon 2013). TNFα interacts with signaling receptors TNFRSF1A (TNFR1) and TNFRSF1B (TNFR2) expressed on the surface of many cell-types including several associated with the reproductive tract. Given the above-mentioned evidence for the involvement of macrophages, it is perhaps not

surprising that TNFα, one of their key cytokine products, has been shown to exert multiple physiological effects on ovarian function including modulatory effects on follicular and luteal steroidogenesis, follicle atresia and luteolysis (Sheldon, et al. 2014, Turner, et al. 2011, Walusimbi and Pate 2013, Wu, et al. 2004).

Similarly, interleukin-6 (IL6), another multifunctional pleiotropic cytokine displaying both pro-inflammatory and anti-inflammatory properties, has been implicated as an intraovarian regulator. Activated macrophages and many other immune cells express IL6, as do most stromal cells (Hunter and Jones 2015). Ovarian TC and GC have both been reported to express IL6 (Bromfield and Sheldon 2011, Liu, et al. 2009, Price and Sheldon 2013, Taylor and Terranova 1995). As with TNFα, IL6 production by macrophages is evoked by various stimuli including activation of toll-like receptors (TLR) by microbial pathogen-associated molecular patterns (PAMPs). Raised levels of other pro-inflammatory cytokines including TNFa and interleukin 1b can further upregulate IL6 production in an autocrine/paracrine manner (Hunter and Jones 2015). In addition to its key role in innate and adaptive immune responses to infection, IL6 has been implicated in various physiological and pathophysiological processes linked with inflammatory responses including metabolic regulation, neuroendocrine control, reproductive dysfunction, insulin resistance and vascular disease (Scheller, et al. 2011, Telleria, et al. 1998).

In the reproductive system, TNFα and IL6 have been reported to exert regulatory effects on ovarian steroidogenesis, angiogenesis and luteolysis; they are also implicated in regulating pregnancy, and parturition (Bornstein, et al. 2004, Franczak, et al. 2012, Galvao, et al. 2013, Sheldon, et al. 2014). It is also evident that subfertility commonly associated with postpartum uterine infections in cattle is associated with inflammatory responses to bacterial PAMPs reaching the ovary and adversely affecting follicular estrogen output and oocyte quality (Sheldon, et al. 2014). In recent years evidence has also accrued to support the concept that chronic 'low grade' inflammation may contribute to the pathogenesis of polycystic ovarian syndrome (PCOS), a common disorder in humans associated with ovarian hyperandrogenism, arrested follicle development and subfertility (Duleba and Dokras 2012, Gonzalez 2012). In this context there has been a resurgence of interest in exploring the contributions of macrophages and inflammatory mediators as intraovarian regulators.

Despite a considerable body of research on the intraovarian actions of cytokines such as TNFα and IL6, most studies have focussed on corpus luteum function and granulosa cells function with relatively attention directed towards theca cells that play a key role in ovarian androgen production, and hence granulosal estrogen output. With this in mind, the present objectives were to (1) generate a transcriptional profile of theca interna and granulosal expression of TNF, IL6 and their receptors (TNFRSF1A, TNFRSF1B, IL6R) during bovine antral follicle development; (2) compare the follicular expression profile of the endothelial cell 'marker' von Willebrand factor (VWF) and the macrophage 'markers' (TLR4, CD68); (3) examine effects of TNFα and IL6 on steroid production and expression of steroidogenesis-related transcripts by theca interna cells cultured under conditions that retain a non-luteinized 'follicular' phenotype; (4) attempt to recapitulate the effects of TNFα and IL6 treatment by co-culturing TC with macrophages, the presumptive source of these cytokines *in vivo*; (5) determine whether direct exposure to bacterial lipopolysaccharide can modulate thecal androgen secretion.

Materials and Methods

Unless stated otherwise, general consumables, chemicals and media were purchased from Sigma UK Ltd. (Poole, Dorset, UK) or Fisher Scientific Ltd. (Loughborough, Leicestershire, UK).

Ovary collection and isolation of granulosa and theca interna layers for gene expression analysis

As described previously (Glister, et al. 2010) bovine ovaries were collected from an abattoir and antral follicles 1-18mm in diameter were dissected out, sorted according to size and their GC, TC layers and follicular fluid recovered for analysis. Individual follicles in the 1-2mm (10 follicles per pool, n=4 pools analysed), 3-4mm (6 follicles per pool; n=5 pools analysed) and 5-6mm (6 follicles per pool; n=5 pools analysed) size categories were combined for further analysis while all follicles >7mm in diameter were processed and analysed individually (n=7-9 per category). On the basis of estrogen:progesterone ratio (E:P ratio) in follicular fluid, follicles in the largest 11-18mm size category were retrospectively subdivided into presumptive healthy large estrogen-active (LEA) follicles (E:P ratio>1) and large estrogen-inactive (LEI), most likely undergoing regression (E:P ratio <1) (see(Glister, et al. 2010). After homogenisation in 0.5ml of Tri reagent GC and TC extracts were stored at -80^{0}C until RNA purification.

Primary TC culture experiments

For *in vitro* experiments, GC and TC pooled from 4-6mm follicles from ~10 ovaries (~50 follicles per culture) were collected as above and further processed as described by Glister et al. (2005) to obtain individual cell suspensions. Only results relating to TC are presented in the current paper. The chemically-defined, serum-free culture medium used throughout was McCoy's 5A supplemented with 10ng/ml insulin (bovine pancreas), 2mM L-glutamine, 10mM Hepes, 5μg/ml apo-transferrin, 5ng/ml sodium selenite, 0.1% (w/v) BSA and 1% (v/v) antibiotic-antimycotic solution. Cells were plated at 75,000 viable cells/well in 96-well plates (Nunclon, Life Technologies Ltd, Paisley, UK) or 0.5 x 10^6 viable cells/well in 24-well plates and cultured for 6 days at 38.5°C. Media were removed every 48h and replaced with fresh media containing treatments (see below). Cell-conditioned medium from the final 48h culture period was stored at –20°C for analysis of androstenedione (A4) and progesterone (P4) concentrations by ELISA. At the end of culture (96 well plates only) viable cell number was determined using neutral red uptake assay (Glister, et al. 2001). In the case of experiments conducted in 24-well plates, cells were lysed using RNeasy lysis buffer (Qiagen) and pooled lysates from 3 replicate wells combined for RNA extraction.

Monocyte-derived macrophages from peripheral blood mononuclear cells (PBMC)

Peripheral blood monocytes were prepared using a method adapted from (Birmingham and Jeska 1980) to incorporate a Histopaque density gradient centrifugation step. Fresh cow blood was collected at a local abattoir in a sterilized 500ml polypropylene bottle containing 40ml of sterile 4% v/w sodium citrate in ultrapure water. Citrated blood was transferred to the lab on ice, transferred into sterile centrifuge tubes and centrifuged at 1200 x g for 25 min at room temperature. The buffy coat was aspirated from the top of the sedimented erythrocyte layer from each tube and pooled into a sterile 50 ml tube. Accuspin tubes (Sigma Ltd) were prepared at room temperature by loading with 15ml of Histopaque-1077 according to the manufacturer's instructions. Briefly 8 ml of buffy coat-enriched aspirate was poured into each Accuspin tube and centrifuged at 1000 x g for 10 min. at room temperature. The top plasma layer was removed and the retained PBMC layer aspirated and transferred to a sterile 15 ml conical centrifuge tube. This tube was topped up with PBS and centrifuged at 300 x g for 10 min. at room temperature. The supernatant was removed and the cell pellet resuspended in 2 ml of PBS. Residual erythrocytes were lysed by adding 4 ml sterile water and mixing gently. After 10 seconds 4ml of 2X PBS was added and mixed briefly to restore isotonicity. The PBMC suspension was centrifuged at 300 x g for 10 min at room temperature, the supernatant was removed and the cell pellet was resuspended in 2ml sterile culture medium (McCoys 5A, 10% (v/v) FCS, 2mM L-glutamine, 1% (v/v) antibiotic-antimycotic solution) for counting (trypan blue; haemocytometer) and plating out in 6-well culture plates at 10^6 cells/ml. After 24h medium was removed and adherent cells (presumptive monocytes) were washed vigorously (x3) with sterile PBS to remove non-attached cells. Thereafter culture medium was changed every 3 days. After day 7 the adherent cells had a macrophage-like morphology and were immunoreactive for the macrophage markers CD68 and MHCII (data not shown); they also showed a marked (>10-fold) upregulation of TNF, IL6 and TLR4 mRNA expression when treated for 4h with bacterial lipopolysacharide (LPS) (data not shown). These monocyte-derived cells, hereafter referred to as macrophages, were used in TC co-culture experiments after 7-10 days of culture. After removing media and washing wells (x2) with PBS, trypsin/EDTA was added to detach cells (~5min) and macrophages were retrieved with the aid of a cell scraper, washed (x2) in PBS and counted. Macrophages were diluted in serum-free TC culture medium and 50,000 cells/ml added to 24-well plates seeded 2 days previously with 0.5 million TC.

Cell culture treatments

Ovine LH (NIADDK oLH-19SIAPP) was provided by the NHPP (Torrance, CA, USA) and human recombinant TNFα and IL6 were purchased from R&D Systems (Abingdon, Oxfordshire, UK). Lipopolysaccharide (LPS; from E.coli 0111:B4; BioExtra grade) was purchased from Sigma (UK) Ltd. Treatments were sterilized using 0.2-μm filters before further dilution in sterile culture medium. In a preliminary dose ranging experiment (data not shown) LH was tested at 0, 10, 100, 500 and 10,000pg/ml and 100pg/ml was shown to give an optimal response in terms of A4 secretion. Cells were treated with TNFα and IL6 at a wide range of concentrations (0.004-50ng/ml) to evaluate the effects on steroid production. This range includes concentrations of TNFa (100-500 pg/ml) and IL6 (400-900 pg/ml) that have been reported in bovine (buffalo) follicular fluid (Boby, et al. 2016) and IL6 concentrations in bovine GC-conditioned media (1-4 ng/ml) (Bromfield and Sheldon 2011). In subsequent experiments to examine effects on gene expression, maximally effective concentrations of TNFa (10 ng/ml) and IL6 (50 ng/ml)

were chosen with the aim of generating robust transcriptional responses. These concentrations are similar to those used in previous in vitro studies on GC (Alpizar and Spicer 1994, Glister, et al. 2014, Salmassi, et al. 2001, Spicer 1998). Treatments (25µl/well) were added after 48 and 96h of culture with an equal volume of blank medium added to control wells. Cultures were terminated at 144h.

Steroid measurements

Concentrations of P4 and A4 in cell-conditioned media were measured using competitive ELISA (Bleach, et al. 2001, Glister, et al. 2013). The detection limit of the P4 assay was 20pg/ml and intra- and inter-plate CVs were 8% and 10% respectively. The detection limit of the A4 assay was 30pg/ml and intra- and inter-plate CVs were 7% and 10% respectively.

RNA isolation, cDNA synthesis and quantitative PCR

Total RNA was isolated from lysates of follicular GC and TC samples using the Tri reagent protocol as described previously (Glister, et al. 2010). For cell culture experiments cell lysates were processed using RNeasy mini kits (Qiagen) according to the manufacturer's protocol. In both cases a DNAse treatment step was included to remove potential genomic DNA contamination from RNA preparations. RNA quantity and quality were evaluated by spectrophotometry at 260/280nm and first strand cDNA was synthesized from 1µg of RNA using the AB High Capacity cDNA synthesis kit (Thermo Fisher Scientific; used according to manufacturer's protocol) in a 20µl reaction primed with random hexamers. Primers (table 1) were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) including BLAST specificity checking to exclude potential amplification of unintended Bos Taurus sequences in the database. Melt-curve analysis and agarose gel electrophoresis was used to verify that each primer pair generated a single amplicon of the predicted size. cDNA template log-dilution curves were used to demonstrate satisfactory PCR efficiency and linearity. PCR assays were carried out in a volume of 14µl, comprising 5µl cDNA template (1:50 dilution), 1µl each forward and reverse primers (final concentration 0.36µM) and 7µl QuantiTect SYBR Green QPCR 2x Master Mix (Qiagen, Crawley, W. Sussex, UK). Samples were processed on an AB StepOne Plus thermal cycler (Thermo-Fisher Scientific) with cycling conditions: 15min at 95°C (one cycle only) followed by 15s at 95°C and 1min at 60°C (40 cycles). The ΔΔCt method (Livak and Schmittgen 2001) was used to compare the relative abundance of each mRNA transcript. Ct values for each transcript in a given sample were first normalized to ß-actin Ct value (uniform across experimental all groups; $P>0.1$). For follicle GC and TC samples ΔCt values for each transcript in a given sample were normalized to the mean ΔCt value for that transcript in all tissue samples. For TC culture experiments the resultant ΔCt values for each treatment replicate was normalized to the mean ΔCt value of the respective vehicle-treated control group. For graphical presentation ΔΔCt values were converted to fold-differences using the formula: fold-difference = $2^{(-\Delta\Delta Ct)}$.

Statistical Analysis

For statistical analysis of steroid secretion results, data from each batch of cells were normalized to vehicle-treated control values (100%) and results presented are amalgamated from 3-4 independent cultures (i.e. 3-4 biological replicates). Quantitative PCR data were analysed as ΔCt values before conversion to fold-difference values used for graphical presentation of results. Statistical analysis was done using one- and/or two-way ANOVA; providing a significant F ratio was obtained, by ANOVA, post-hoc pairwise comparisons were made using Fisher's protected least significant difference (PLSD) test.

Results

Expression of TNF, IL6 and their receptors in granulosa and theca interna layers of developing antral follicles

The relative abundance of TNF mRNA increased 3-4-fold over the course of antral follicle development in both TC and GC (Fig. 1A). Overall, the relative abundance of TNF mRNA was higher ($P<0.01$) in TC than GC (fig. 1) with greatest expression in TC of LEA follicles (~9-fold higher than in TC of 1-2mm follicles). The relative abundance of TNFRSF1A mRNA in TC showed a progressive 10-fold increase between 1-2mm and 11-18mm follicle size categories ($P<0.0001$; Fig 1B). In contrast expression level in GC was relatively uniform. There were no significant cell-type or follicle stage-related differences in the abundance of TNFRSF1B mRNA that was only detected at low levels in these samples (Fig. 1C)

The relative abundance of IL6 mRNA was greatest in LEI follicles being ~5-fold higher than in corresponding LEA follicles of equivalent size ($P<0.001$) but there was no significant difference between GC and TC (Fig 1D). In contrast, the abundance of IL6R mRNA was greater in GC compared with TC at all follicle stages, particularly in large (11-18mm) follicles, regardless of their estrogen-active status (Fig 1E). GC expression of IL6R increased ~10-fold between 1-2mm and 11-18mm size categories ($P<0.05$). TC expression of IL6R was ~2-fold higher in 11-18mm follicles than in 1-2mm follicles.

Expression of putative endothelial cell (VWF) and macrophage (CD68, TLR4) 'markers' in granulosa and theca interna layers of developing antral follicles

Fig. 1F shows that VWF mRNA abundance was ~50-fold higher in TC than GC. Expression levels increased ~3-fold in both compartments between 1-2 and 9-10 mm in diameter ($P<0.05$) but were lower in 11-18mm follicles than in 9-10mm follicles ($P<0.05$). Overall, expression levels of CD68 (Fig. 1G) and TLR4 (Fig. 1H) were both higher in TC than GC. Thecal CD68 and TLR4 expression increased ~4-fold between 1-2mm and 9-10mm ($P<0.05$) but was lower in LEA follicles than in 9-10mm follicles and LEI follicles ($P<0.05$). More variable profiles were seen in GC with CD68 expression being lower in 7-8mm follicles than at all other stages ($P<0.05$); TLR4 expression in GC was higher in LEI follicles than in 1-4mm and 7-8mm follicles ($P<0.05$).

Effect of TNFα on basal and LH-induced production of androstendione and progesterone and on viable cell number

Treatment of cells with LH at 100pg/ml had a positive effect on A4 secretion ($P<0.0001$). At a 10-fold higher LH concentration (1000pg/ml) no increase in A4 secretion was observed but P4 secretion was markedly (~100-fold; $P<0.0001$) increased

reflecting cellular luteinisation (Fig. 2). TNFα completely suppressed LH-induced A4 secretion in a dose dependant manner (P=0.007) with an IC_{50} ~80 pg/ml. (Fig. 2). TNFα had no effect on P4 level in LH treated cells, however under basal conditions P4 secretion was increased by the highest 3 doses of TNFα (>2ng/ml; P<0.05). Viable cell number was decreased dose-dependently by both LH (~30%; P<0.0004) and TNFα (~50%; P<0.0001).

Effect IL6 on basal and LH-stimulated production of androstendione and progesterone and on viable cell number

Treatment of cells with IL6 dose-dependently suppressed basal and LH-induced A4 secretion (P=0.007) but only by about 60% at the highest concentration tested (50ng/ml) (Fig. 2). Overall, IL6 had a small though significant (P<0.005) inhibitory effect on basal and LH-induced P4 secretion, the response being most pronounced (~10-fold suppression) in cells exposed to the high (luteinizing) dose-level of LH. This effect was evident with IL6 concentrations as low as 20 pg/ml. IL6 had no effect on viable cell number.

Effect of TNFα on thecal expression of steroidogenesis-related transcripts and on NOS2 and NOS3

TC cultures were scaled up in 24 well plates to provide a sufficient number of cells for RNA extraction and gene expression analysis. One dose-level of each treatment (100pg/ml LH, 10ng/ml TNFα, 50ng/ml IL6) was selected based on optimal responses in the dose-response experiment (see Fig 2). Relative mRNA expression levels of target genes were normalized to the house keeping gene ACTB, which had uniform Ct values in control and treated cells. As above A4 secretion was increased significantly by LH treatment (100 pg/ml) and this response was abolished by TNFα treatment (Fig 3). Moreover, this was accompanied by a profound (~50-fold) reduction in the abundance of CYP17A1 and INSL3 transcripts, under both basal and LH stimulated conditions (Fig 3). TNFα significantly reduced expression of two other genes involved in thecal steroidogenesis; LHCGR transcript abundance was reduced by ~50-fold and HSD3B1 by ~50% under both basal and LH-stimulated conditions (Fig. 4). There was no effect on expression of NR5A1, STAR, CYP11A1 or HSD17B1. Under basal and LH-stimulated conditions TNFα promoted a 10-fold upregulation of NOS2 mRNA expression whilst downregulating NOS3 expression (Fig. 4). While there was no significant effect of TNFα on P4 secretion in LH treated cells, TNFα had a stimulatory effect on P4 secretion under basal conditions (data not shown) in agreement with the finding in the above dose response experiment (see Fig 2). STAR expression tended to be higher in cells treated with TNFα under basal conditions but the difference was not significant.

Effect of IL6 on thecal expression of steroidogenesis-related transcripts and on NOS2 and NOS3

Treatment with IL6 reduced LH-induced A4 secretion concomitantly with a suppression of CYP17A1 transcript abundance but did not affect INSL3 expression (Fig. 4). IL6 also had a modest inhibitory effect on P4 secretion under both basal and LH-stimulated conditions (data not shown), in agreement with the findings from the dose-response experiment. In addition, IL6 reduced STAR mRNA abundance under basal conditions and LHCGR mRNA abundance under LH-stimulated conditions but did not affect any of the other steroidogenic pathway-related genes studied. In contrast to TNFα, IL6 did not alter thecal NOS2 or NOS3 expression.

Effect of macrophages on thecal androgen secretion and gene expression

Since macrophages are a prominent source of pro-inflammatory cytokines such as TNFα and IL6, we examined the effect of co-culturing TC with macrophages on androgen secretion and steroidogenesis-related gene expression. As observed with TNFα and IL6 treatment, exposure of TC to macrophages suppressed LH-induced A4 secretion concomitantly with a reduction in CYP17A1, INSL3 and LHCGR transcript abundance (Fig. 5). In addition, macrophages suppressed STAR, CYP11A1 and HSD3B1 expression under both basal and LH-stimulated conditions but did not affect NOS2 or NOS3 expression (Fig. 5).

Direct effect of LPS and TLR4 inhibitor on thecal androgen secretion

To examine whether cultured TC are capable of sensing and responding directly to bacterial PAMPs cultured TC were treated with LPS in the presence and absence of an inhibitor of TLR4. Fig. 6 shows that treatment of TC with LPS suppressed LH-stimulated A4 secretion ($P<0.05$). Co-treatment with TLR4 inhibitor (TAK242) reduced the suppressive effect of LPS on A4 secretion ($P<0.05$) but had no effect on A4 secretion in the absence of LPS.

Discussion

The first part of the study generated quantitative mRNA expression profiles for two key pro-inflammatory cytokines (TNF, IL6) and their signaling receptors (TNFRSF1A, TNFRSF1B, IL6R) in granulosal and theca interna compartments of developing bovine antral follicles. The finding that all five transcripts were detected in all samples, coupled with the observation of significant cell-type and follicle stage-dependent differences in transcript abundance, supports intra-follicular actions of locally produced TNFα and IL6. Thus, both TC and GC are a source and target of these cytokines, as well as being responsive to circulating cytokines from extra-ovarian tissues, such as those arising from inflammatory reactions to bacterial infections of the post-partum uterus or mammary gland in dairy cattle (Bromfield, et al. 2015, Lavon, et al. 2011, Sheldon, et al. 2014). The finding of broadly similar TNF and IL6 mRNA expression levels in GC and TC layers is consistent with previous evidence from several species that follicular somatic cells are capable of producing these cytokines. Many studies have documented the ability of GC from several species to express and/or secrete TNFα and IL6 including bovine (Bromfield and Sheldon 2011, Glister, et al. 2014, Price, et al. 2013, Price and Sheldon 2013, Zolti, et al. 1990), human (Adams, et al. 2016, Ibrahim, et al. 2016) and mouse (Liu, et al. 2009) but there have been relatively few reports pertaining to TC (Jatesada, et al. 2013, Loret de Mola, et al. 1996, Taylor and Terranova 1995).

The finding of increased expression of TNF and IL6 mRNA during bovine antral follicle development supports an earlier report (Zolti, et al. 1990) that bovine GC secrete TNFα protein and that its level in follicular fluid is higher in peri-ovulatory follicles than mid-cycle follicles. TNFα inhibited TC A4 secretion with an IC_{50} value of ~80 pg/ml, well within the concentration range observed in buffalo follicular fluid (Boby, et al. 2016). Similarly, IL6 reduced P4 secretion at concentrations as low as 20 pg/ml but only suppressed A4 secretion at much higher concentrations. TNFα and IL6 have also been detected in human follicular fluid (Altun, et al. 2011, Baskind, et al. 2014, Lee, et al. 2000, Wang, et al. 1992) with higher IL6 levels evident during the peri-ovulatory period (Baskind, et al. 2014).

With regard to the cytokine receptors we examined in developing antral follicles, in smaller follicles (1-6mm) TNFRSF1A was more highly expressed in TC than GC perhaps suggesting they have greater thecal responsiveness to TNFα. However, while TC expression of TNFRSF1A remained relatively uniform throughout follicle development, GC expression increased progressively (~7-fold) from 1-2 mm to 11-18 mm follicles, implying a greater GC responsiveness to TNFα in large follicles. However, an earlier report based on evaluation of radiolabelled TNFα binding to membrane fractions (Sakumoto, et al. 2003) did not detect any difference in TNFα receptor density between TC and GC from small versus preovulatory follicles. The relative abundance of TNFRSF1B was much lower than for TNFRSF1A and no significant effect of cell-type or follicle stage was recorded. IL6R was much more highly expressed in GC than in TC, particularly in large follicles and this lends support to a previous study documenting a more active role of IL6 in GC than in TC (Breard, et al. 1998).

In an attempt to evaluate potential changes in the relative numbers of macrophages and endothelial cells in the theca interna and/or granulosal layers during follicle development, mRNA expression levels of two putative macrophage 'markers' (CD68, TLR4) and an endothelial; cell 'marker' (VWF) were also determined. As anticipated, VWF expression was much higher (~50-fold) in the vascularized TC compartment than in the avascular GC compartment and TC expression increased ~3-fold during follicle growth from 1-2 to 9-10mm consistent with increased density of capillaries. However, a comparable increase in VWF mRNA observed in the GC compartment is difficult to reconcile with the supposedly avascular nature of this compartment and questions the utility of VWF transcript as a specific endothelial cell marker. This limitation is supported by several microarray studies documenting VWF expression in bovine GC (Glister, et al. 2014, Hatzirodos, et al. 2014, Khan, et al. 2016) Also, the majority of cultured human and mouse GC reportedly express VWF protein (Antczak and Van Blerkom 2000).The same caveat applies to interpretation of the CD68 and TLR4 expression profiles in these samples since microarray studies have clearly documented expression of both transcripts in many other cell-types including bovine GC (Glister, et al. 2014, Hatzirodos, et al. 2014, Khan, et al. 2016), TC (Glister, et al. 2013, Hatzirodos, et al. 2015) and vascular endothelial cells (Busnadiego, et al. 2013). Moreover, bovine GC preparations from healthy large antral follicles are reportedly devoid of macrophages and other immune cells, yet express TLR4 and other TLRs (Herath, et al. 2007, Price, et al. 2013). Nonetheless, significant cell-type and follicle stage-related differences in CD68 and TLR4 mRNA abundance were seen with higher overall levels of both in TC than GC, and with increased expression accompanying follicle growth between 1-2 mm and 9-10 mm. Interestingly, levels in TC then declined in large E2-active follicles but not in large E2-inactive follicles. The extent to which these changes can be considered to reflect changes in tissue density of macrophages is largely unknown and would require a detailed quantitative immunohistological and/or in situ hybridization study in parallel with gene expression profiling.

Treatment of TC with TNFα potently suppressed LH-induced androgen production (IC_{50} ~80pg/ml) with concomitant reductions in expression of CYP17A1, LHCGR, INSL3 and HSD3B1. Basal expression levels of these transcripts were also reduced by TNFα treatment. These findings accord with earlier reports for bovine TC (Spicer 1998) and rat theca-interstitial cells (Zachow, et al. 1993) that TNFα inhibits LH-induced androgen secretion. However, the magnitude of the effect we observed (~100% suppression) was substantially greater than the <50% suppression observed previously

(Spicer 1998) likely reflecting the fact that our TC were cultured with a complete absence of serum, whereas the earlier study cultured cells in serum-supplemented medium for the first 2 days. In our experience TC have the propensity to luteinize when cultured with serum, as reflected by diminished androgen output and greatly increased progesterone output. Another study (Williams, et al. 2008) also observed a relatively modest effect of TNFα on androgen secretion by bovine TC; in contrast to the present study, the cells were not provided with LH stimulation, likely explaining the low responsiveness.

Interestingly, under both basal and LH-treated conditions, expression of the classical TNFα-responsive gene, NOS2 (iNOS), was markedly upregulated by TNFα treatment while NOS3 (eNOS) expression was downregulated. Treatment of bovine TC with BMP6 was also found to enhance NOS2 expression whilst suppressing androgen secretion and CYP17A1 and INSL3 expression (Glister, et al. 2013). NOS2 mRNA level was reportedly higher in GC of growing dominant bovine follicles compared to subordinate follicles (Zamberlam, et al. 2011) and this accords with the present observation that follicular TNF mRNA expression level was maximal in TC of large estrogen-active follicles which showed high expression levels of TNFRSF1A in both TC and GC layers. In IVF patients, high levels of nitric oxide in follicular fluid have been associated with reduced E2 production and diminished oocyte quality (Vignini, et al. 2008) suggesting a possible association with increased TNFα signaling. TNFα has also been shown to suppress FSH-induced estradiol secretion and CYP19A1 expression by GC (Glister, et al. 2014, Kaipia, et al. 1996, Sakumoto, et al. 2003, Spicer 1998, Williams, et al. 2008). With regard to the hypothesis linking chronic low-grade inflammation to the development of PCOS and associated hyperandrogenism (Duleba and Dokras 2012, Gonzalez 2012), the present findings are counter-intuitive since pro-inflammatory cytokines like TNFa might be expected to enhance rather than suppress thecal androgen production as clearly shown here.

Given the observation that IL6 and its receptor are expressed by both TC and GC throughout bovine antral follicle development, we also examined the effect of IL6 on TC steroidogenesis. An inhibitory effect of IL6 was seen on LH-induced secretion of both A4 and P4 although the potency of IL6 in suppressing A4 secretion was much less than that of TNFα. In contrast, IL6 at concentrations as low as 20 pg/ml reduced thecal P4 secretion, indicating effects at concentrations well within the range observed in follicular fluid and GC-conditioned culture medium (Boby, et al. 2016, Bromfield and Sheldon 2011). The inhibition of A4 secretion elicited by the much higher (likely supra-physiological) concentration of IL6 (50 ng/ml) used in our gene expression experiment was accompanied by a significant down regulation of CYP17A1, LHCGR and STAR mRNA abundance but there was no clear effect on expression of the other genes examined including INSL3, NOS2 and NOS3 that were clearly modulated by TNFα. Earlier studies found no effect of IL6 on androgen secretion by rat theca-interstitial cells (Hurwitz, et al. 1991) or in vitro perfused rat ovary (Van der Hoek, et al. 1998). However, IL6 has been reported to exert an inhibitory action on GC oestrogen secretion (Alpizar and Spicer 1994, Salmassi, et al. 2001, Spicer 1998, Tamura, et al. 2000). Further studies are needed to unravel these apparently complex differential actions of TNFα and IL6 on thecal steroidogenesis.

Macrophages, particularly when activated (e.g. by microbial PAMP exposure), are a prominent source of pro-inflammatory cytokines including TNFα, IL6 and IL1b (Plowden, et al. 2004). In addition to a resident macrophage population, monocytes are

known to infiltrate the ovary in a cyclic manner where they differentiate into macrophages (Figueroa, et al. 2012, Wu, et al. 2004). Evidence supports a role for macrophages in follicle atresia, follicular-luteal transition and luteal regression (reviews: (Walusimbi and Pate 2013, Wu, et al. 2004) but their potential involvement in the regulation of orderly follicle growth and steroidogenesis under normal physiological conditions remains unclear. Here, we showed that co-culturing TC with monocyte-derived macrophages blocked LH-induced androgen secretion, accompanied by a reduction in expression of CYP17A1 and several other steroidogenesis-related transcripts including LHCGR, STAR, CYP11A1, HSD3B1 and INSL3. Notably, INSL3 was recently shown to have a positive role in thecal A4 secretion (Glister, et al. 2013, Satchell, et al. 2013) so the inhibitory effect of both macrophages and TNFa might be mediated, at least in part, by downregulation of INSL3 expression leading to loss of CYP17A1 expression. Alternately, downregulation of LHCGR leading to loss of LH sensitivity could explain the decline in LH-dependent CYP17A1 expression and androgen production since LHCGR expression was inhibited by all three treatments (macrophages, TNFα, IL6).

Both GC (Bromfield and Sheldon 2011, Herath, et al. 2007, Price, et al. 2013, Price and Sheldon 2013, Shimizu, et al. 2012) and TC (Magata, et al. 2014a, Taylor and Terranova 1995, Williams, et al. 2008) have been shown to express toll-like receptors (TLR) potentially enabling them to detect and respond to bacterial PAMPs (e.g. TLR4 binding to LPS) leading to production of pro-inflammatory cytokines, in a manner akin to macrophages and other immunity-related cells. Indeed, LPS has been shown to promote IL6 and TNF expression/secretion and inhibit CYP19A1 expression and oestradiol secretion by cultured bovine GC (Herath, et al. 2007, Price, et al. 2013, Price and Sheldon 2013). The present findings confirm TLR4 mRNA expression in follicular theca interna layer and GC layers. Moreover, we show herein a direct inhibitory action of LPS on LH-induced androgen secretion by bovine TC that was attenuated by co-treatment with a TLR4 blocker (TAK242). Magata et al. (2014a) also reported an LPS-induced inhibition of thecal androgen production although another bovine study (Williams, et al. 2008) found no effect. Interestingly, LPS and another PAMP from gram-positive bacteria, peptidoglycan, exerted an additive suppressive effect on thecal androgen production (Magata, et al. 2014b). LPS was also reported to inhibit LH-induced androgen and progesterone secretion by rat theca-interstitial cells (Taylor and Terranova 1995).

In summary, cell-type and follicle stage-dependent differences in mRNA expression of TNF, IL6 and their receptors were found in granulosal and theca interna layers of developing bovine antral follicles. The study confirms and extends previous observations regarding the ability of these cytokines to modulate thecal steroidogenesis and demonstrates that macrophage co-culture, like TNFα and IL6 treatment, can suppress thecal androgen secretion and inhibit expression of CYP17A1 and LHCGR. Finally, we provide confirmatory evidence that bovine theca interna cells are directly responsive to bacterial LPS and that this also attenuates LH-dependant androgen secretion. In an *in vivo* context, the findings provide further supporting evidence for the view that both macrophages and follicular somatic cells contribute to the 'local' generation of inflammatory mediators in response to either physiological (i.e. ovarian cycles) or pathophysiological (i.e. bacterial infections) events that can, in turn, exert powerful modulatory actions on ovarian steroidogenesis at both the theca and granulosa cell level.

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FIGURES

Fig 1: Relative abundance of mRNA transcripts for (A) TNF, (B) TNFRSF1A, (C) TNFRSF1B, (D) IL6, (E) IL6R, (F) VWF, (G) CD68 and (H) TLR4 in GC and TC compartments of developing bovine antral follicles. Follicles in the 11-18mm size class have been subdivided on the basis of E2 to P4 ratio (E: P ratio) as "E2-active" (E: P ratio>1) or "E2-inactive" (E: P ratio<1). Values are mean ±SEM and summarized two-way ANOVA results are shown. Results of post-hoc tests comparing different follicle categories are indicated by uppercase (TC) and lowercase (GC) letters; for each cell-type means without a common letter are significantly different ($P < 0.05$).

Fig 2: Effect of TNFα and IL6 on basal and LH-induced secretion of (A) A4; (B) P4 and on (C) viable cell number. Values are mean ±SEM of normalized data from n=4 independent cultures. Summarized two-way ANOVA results are shown.

Fig. 3: Effect of TNFα on basal and LH-dependent A4 secretion and expression of steroidogenesis-related transcripts and NOS2/NOS3 mRNA by cultured bovine theca interna cells. Values are means ± SEM based on 4 independent cultures; bars without a common letter are significantly different.

Fig. 4: Effect of IL6 on basal and LH-dependent A4 secretion and expression of steroidogenesis-related transcripts and NOS2/NOS3 mRNA by cultured bovine theca interna cells. Values are means ± SEM based on 4 independent cultures; bars without a common letter are significantly different.

Fig. 5: Effect of co-culturing theca interna cells with macrophages on basal and LH-dependent A4 secretion and expression of steroidogenesis-related transcripts and NOS2/NOS3 mRNA by bovine theca interna cells. Values are means ± SEM based on 3 independent cultures; bars without a common letter are significantly different.

Fig. 6: Effect of lipopolysaccharide (LPS) in the presence and absence of TLR4 inhibitor (TAK242) on basal and LH-induced A4 secretion by bovine theca interna cells. Values are means ± SEM based on 3 independent cultures; bars without a common letter are significantly different.

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798

FIG 1

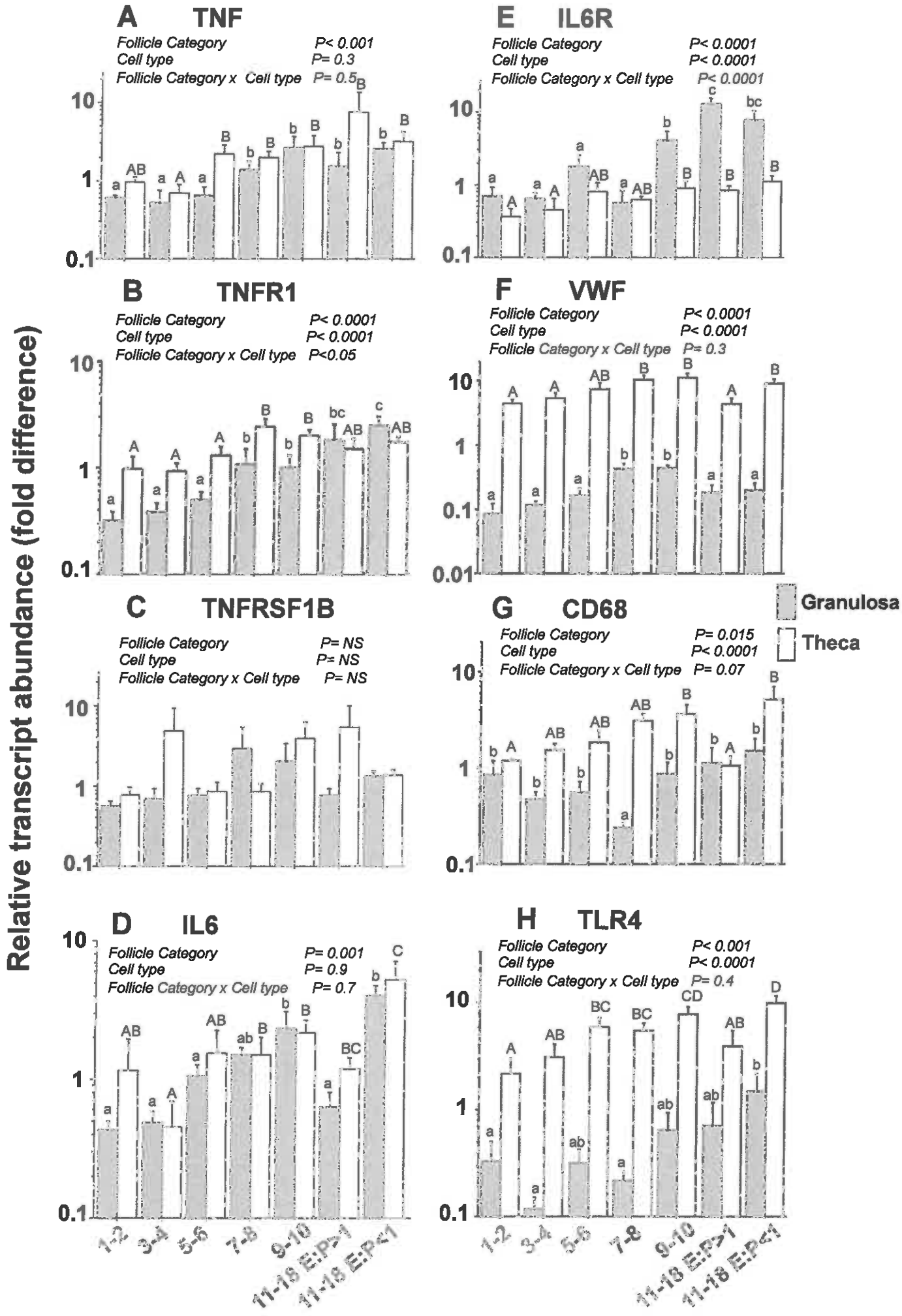

A TNF
Follicle Category P< 0.001
Cell type P= 0.3
Follicle Category x Cell type P= 0.5
E IL6R
Follicle Category P< 0.0001
Cell type P< 0.0001
Follicle Category x Cell type P< 0.0001
B TNFR1
Follicle Category P< 0.0001
Cell type P< 0.0001
Follicle Category x Cell type P<0.05
F VWF
Follicle Category P< 0.0001
Cell type P< 0.0001
Follicle Category x Cell type P= 0.3
C TNFRSF1B
Follicle Category P= NS
Cell type P= NS
Follicle Category x Cell type P= NS
G CD68
Follicle Category P= 0.015
Cell type P< 0.0001
Follicle Category x Cell type P= 0.07
Granulosa
Theca
D IL6
Follicle Category P= 0.001
Cell type P= 0.9
Follicle Category x Cell type P= 0.7
H TLR4
Follicle Category P< 0.001
Cell type P< 0.0001
Follicle Category x Cell type P= 0.4
1-2
3-4
5-6
7-8
9-10
11-18 E:P>1
11-18 E:P<1
Relative transcript abundance (fold difference)
Follicle diameter (mm)

FIG 2

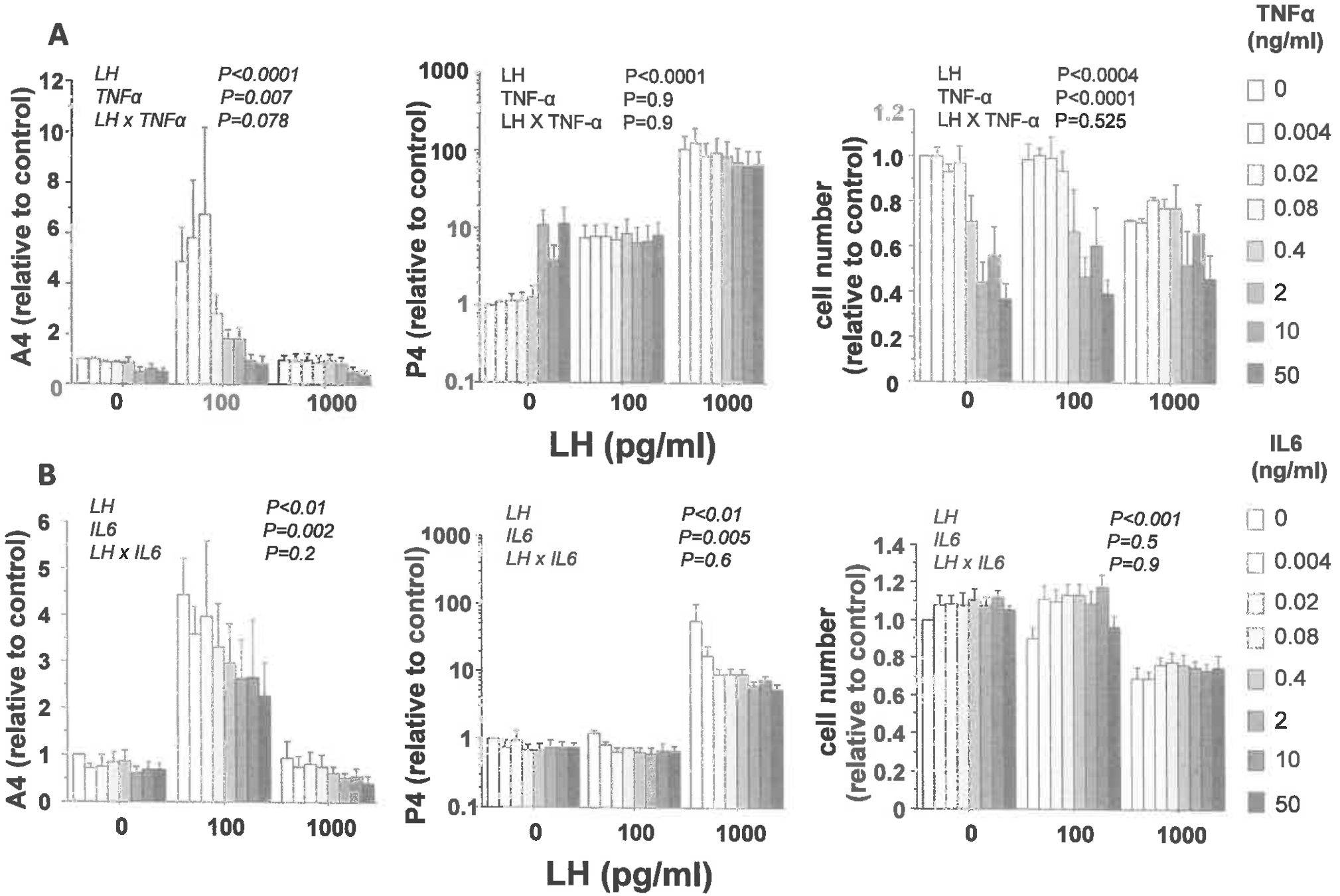

A
LH
TNFα
LH x TNFα
P<0.0001
P=0.007
P=0.078
A4 (relative to control)
P4 (relative to control)
LH
TNF-α
LH X TNF-α
P<0.0001
P=0.9
P=0.9
cell number (relative to control)
P<0.0004
P<0.0001
P=0.525
TNFα (ng/ml)
0
0.004
0.02
0.08
0.4
2
10
50
LH (pg/ml)
B
LH
IL6
LH x IL6
P<0.01
P=0.002
P=0.2
P<0.01
P=0.005
P=0.6
P<0.001
P=0.5
P=0.9
IL6 (ng/ml)

FIG 3

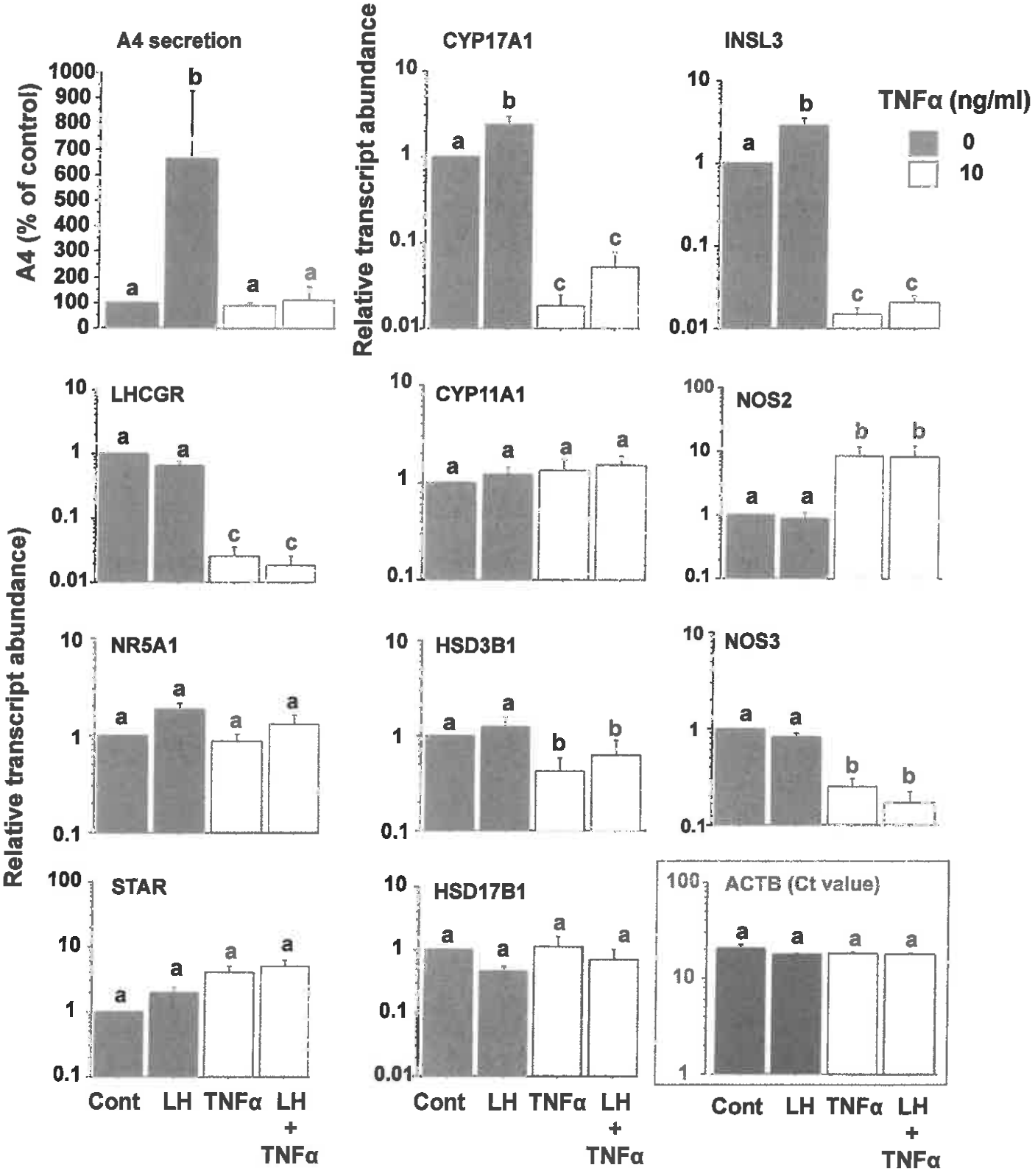

A4 secretion
A4 (% of control)
1000
900
800
700
600
500
400
300
200
100
0
CYP17A1
INSL3
Relative transcript abundance
10
1
0.1
0.01
TNFα (ng/ml)
0
10
LHCGR
CYP11A1
NOS2
100
NR5A1
HSD3B1
NOS3
STAR
HSD17B1
ACTB (Ct value)
Relative transcript abundance)
Cont
LH
TNFα
LH + TNFα

FIG 4

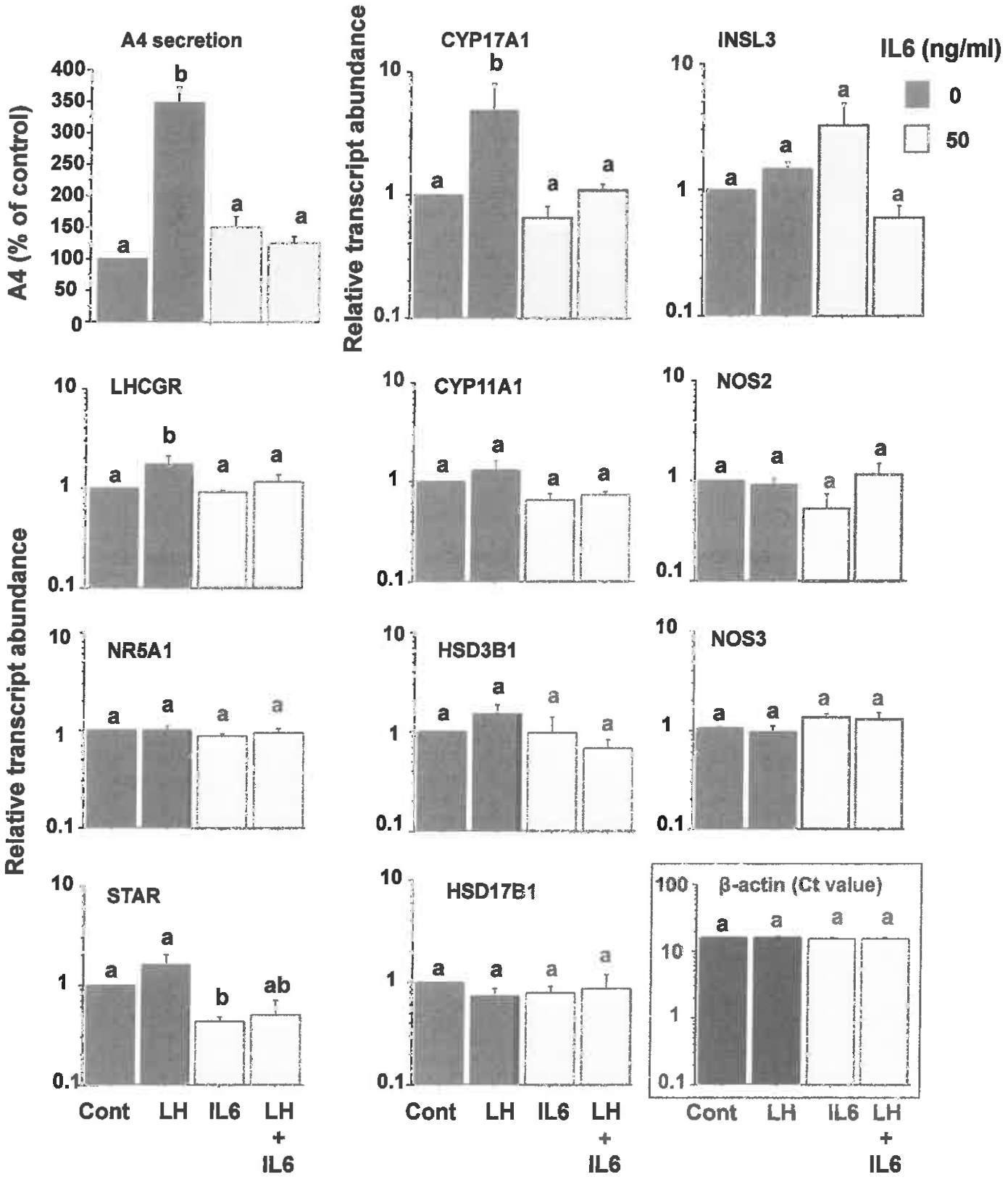

A4 secretion
A4 (% of control)
400
350
300
250
200
150
100
50
0
CYP17A1
Relative transcript abundance
INSL3
IL6 (ng/ml)
0
50
LHCGR
CYP11A1
NOS2
NR5A1
HSD3B1
NOS3
STAR
HSD17B1
β-actin (Ct value)
Relative transcript abundance
10
1
0.1
100
a
b
ab
Cont
LH
IL6
LH + IL6

FIG 5

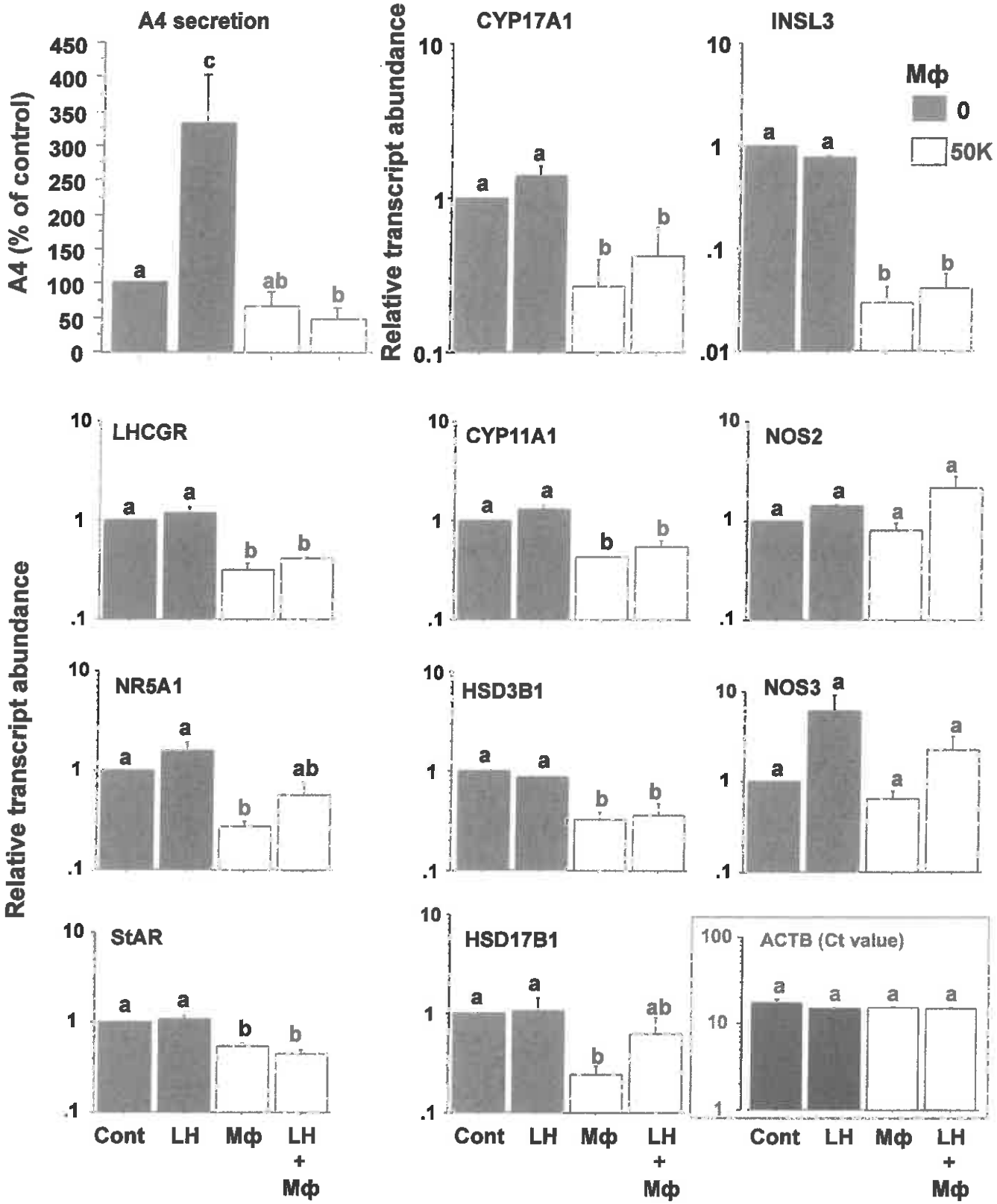
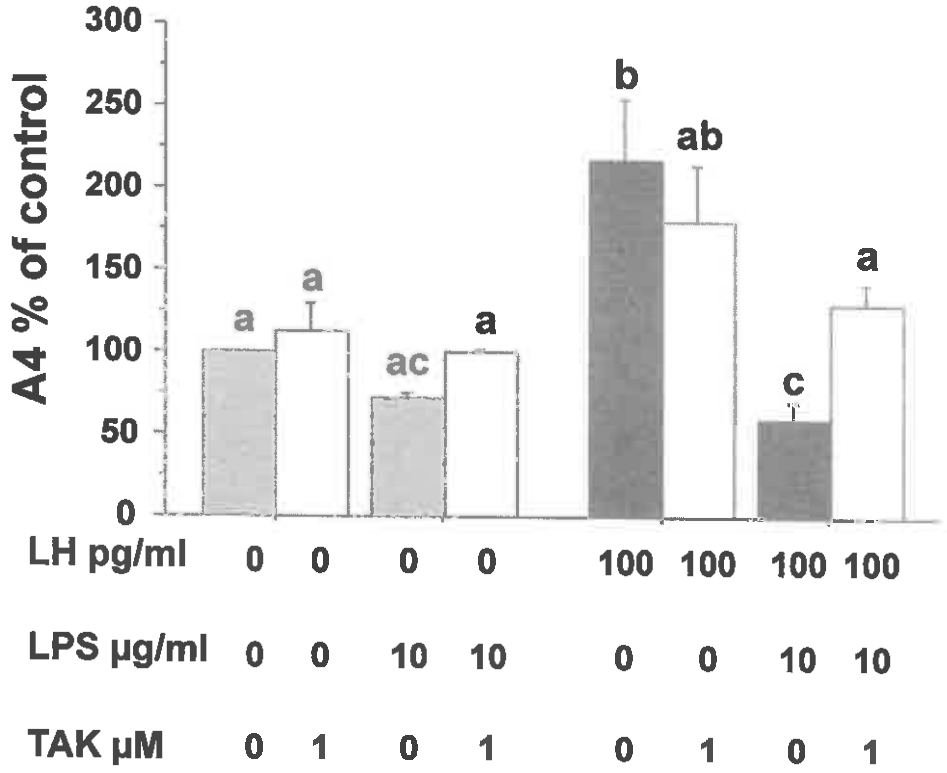

A4 secretion
A4 (% of control)
450
400
350
300
250
200
150
100
50
0
a
c
ab
b
CYP17A1
Relative transcript abundance
10
1
0.1
a
a
b
b
INSL3
10
1
.1
.01
a
a
b
b
Mφ
0
50K
LHCGR
10
1
.1
a
a
b
b
CYP11A1
10
1
.1
a
a
b
b
NOS2
10
1
.1
a
a
a
a
NR5A1
10
1
.1
a
a
b
ab
HSD3B1
10
1
.1
a
a
b
b
NOS3
10
1
.1
a
a
a
a
StAR
10
1
.1
a
a
b
b
HSD17B1
10
1
.1
a
a
b
ab
ACTB (Ct value)
100
10
1
a
a
a
a
Cont
LH
Mφ
LH + Mφ
Relative transcript abundance

FIG 6


A4 % of control
300
250
200
150
100
50
0
a
a
ac
a
b
ab
c
a
LH pg/ml 0 0 0 0 100 100 100 100
LPS µg/ml 0 0 10 10 0 0 10 10
TAK µM 0 1 0 1 0 1 0 1

Target	Accession number	Forward primer 5' to 3'	Reverse primer 5' to 3'	Amplicon size (bp)
NR5A1	S45997.1	CGGGCTACCACTACGGGCTGC	CGGGCTACCACTACGGGCTGC	125
LHCGR	NM_174381.1	ATTGCCTCAGTCGATGCCCAGACC	AAAAAGCCAGCCGCGCTGC	92
STAR	NM_174189	TTTTTTCCTGGGTCCTGACAGCGTC	ACAACCTGATCCTTGGGTTCTGCACC	103
CYP11A1	NM_176644	CAGTGTCCCTCTGCTCAACGTCC	TTATTGAAAATTGTGTCCCATGCGG	99
HSD3B1	NM_174343.2	GCCACCTAGTGACTCTTTCCAACAGCG	TGGTTTTCTGCTTGGCTTCCTCCC	111
HSD17B1	NM_001102365.1	CGCATATTGGTGACCGGGAGCATA	AATCGCCAGACTCTCGCACAAACC	108
CYP17A1	NM_174304	GACAAAGGCACAGACGTTGTGGTCA	TGATCTGCAAGACGAGACTGGCATG	301
INSL3	NM_174365	TCTGTCCCCACTGAATCCTCCTGG	GGGTTTCATGGTGCTGTGTGGC	102
TNF	AF348421.1	GAGGTGCTCTCCGAGAAAGCAGGG	CGATTACCCCGAAGTGCAGCAGG	127
IL6	NM_173923.2	AGCGCCTTCACTCCATTCGC	TGAAATCTTCTCCCAGGGACCCG	91
TNFR1	U90937.1	CCCAATGGCACAGTGAATATCCCC	GGCAAAGCCCGAAGACAATCACC	220
TNFRSF1B	NM_001040490.2	ATCGCATCTGCACCTGCAAGCC	TGCAGTTCCTGGTTTGGCCACG	122
IL6R	NM_001110785.1	AGGGATCAGATGACAGGCTCGC	AACATCCACCACCAAGCGCACG	150
NOS2	NM_001076799	AAGCCGTGTTCTTCGCCTCG	AGGCACAGCTGAACAAAGCCCC	135
NOS3	NM_181037.3	CCACATCAAGTACGCCACCAACCG	ACCAGCTGGCTGTTCCAGATCC	108
TLR4	NM_174198.6	CCCAGCACTGCTTTGAATAGGGGC	ATAACCCTCTGTCTGTGCCGGC	106
CD68	NM_001045902.1	AGGTACCCATTCCCACTTGCTCC	AATGTCCACTGCACTGCCTGGG	147
VWF	NM_001205308.1	GGGGAGGACCTGCAGATAGA	GGAAGTCGTCCCTCTGGTTG	124
ACTB	NM_173979.3	ATCACCATCGGCAATGAGCGGTTC	CGGATGTCGACGTCACACTTCATGA	128

Table 1: List of primers used for quantitative RT-PCR