Neonatal immune activation depletes the ovarian follicle reserve and alters ovarian acute inflammatory mediators in neonatal rats

Running title: Neonatal immune stress depletes early follicles

Summary Sentence: Neonatal immune activation acutely impacts immune-mediated early ovarian development, depleting the primordial follicle pool and upregulating inflammatory mediators.

Key Words: Early life immune stress, lipopolysaccharide, inflammation, cytokines, follicular development, oocyte development, developmental origins of health and disease.

Erin. A. Fuller [1, †], Luba Sominsky [2], Jessie. M. Sutherland [3], Kate. A. Redgrove [3], Lauren Harms[1], Eileen. A. McLaughlin [3, 4], Deborah. M. Hodgson [1].

[1] Laboratory of Neuroimmunology, Priority Research Centre for Brain and Mental Health Research, School of Psychology, University of Newcastle, Callaghan, NSW 2308, Australia

[2] School of Health and Biomedical Sciences, RMIT University, Melbourne, VIC 3083, Australia

[3] School of Environmental and Life Sciences, Priority Research Centre in Chemical Biology, University of Newcastle, Callaghan, NSW 2308, Australia

[4] School of Biological Sciences, University of Auckland, Auckland, 1010, NZ

†Corresponding author Erin.Fuller@newcastle.edu.au, University of Newcastle, University Drive, Callaghan, NSW, 2380

Declaration of interest

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Abstract

Normal ovarian development is crucial for female reproductive success and longevity. Interruptions to the delicate process of initial folliculogenesis may lead to ovarian dysfunction. We have previously demonstrated that an early life immune challenge in the rat, induced by administration of lipopolysaccharide (LPS) on postnatal day (PND) 3 and 5, depletes ovarian follicle reserve long term. Here, we hypothesised that this neonatal immune challenge leads to an increase in peripheral and ovarian inflammatory signalling, contributing to an acute depletion of ovarian follicles. Morphological analysis of neonatal ovaries indicated that LPS administration significantly depleted PND 5 primordial follicle populations and accelerated follicle maturation. LPS exposure upregulated circulating interleukin 6 (IL6), tumour necrosis factor alpha (TNFa), and C-reactive protein (CRP) on PND 5, and upregulated ovarian mRNA expression of *Tnfa,* mitogen-activated protein kinase 8 (*Mapk8/Jnk1*), and growth differentiation factor 9 (*Gdf9)* (p <0.05). Mass Spectrometry and cell signalling pathway analysis indicated

upregulation of cellular pathways associated with acute phase signalling, and cellular survival and assembly. Apoptosis assessed by TUNEL indicated significantly increased positive staining in the ovaries of LPS treated neonates. These findings suggest that increased proinflammatory signalling within the neonatal ovary may be responsible for the LPS-induced depletion of the primordial follicle pool. These findings also have implications for female reproductive health, as the ovarian reserve is a major determinate of female reproductive longevity.

Introduction

Despite significant medical advances, idiopathic infertility and the prevalence of reproductive disorders in younger female cohorts is increasing [1-4]. As the fundamentals of reproductive health and longevity are established in early life, the pathogenesis of female reproductive dysfunction may have developmental roots. Abnormalities occurring during this critical period of development may lead to sustained ovarian pathophysiology, including premature ovarian failure (POF) and other fertility issues [5-7]. Recent evidence indicates that the female reproductive system is sensitive to early life stressors, such as xenobiotics, infections, and malnutrition, which can perturb reproductive development and negatively impact long term fertility levels [8-12].

Mammalian female reproductive health and success is reliant on the normal establishment of the non-renewing ovarian primordial follicle reserve, which is the foundation of all future follicles and determines the reproductive lifespan [as reviewed in 13, 14]. This initial folliculogenesis occurs prenatally in humans, however finalises during the 1st postnatal week in rodents. Early follicles containing oocytes ultimately

develop from the primordial stage through to the ovulatory stage, until the reserve is diminished and menopause, or senescence in rats, occurs [15]. Initial folliculogenesis is governed by numerous mechanisms which remain to be fully elucidated, however current evidence indicates that complex interactions of chemokines, cytokines, neurotrophins, growth factors, and transcription factors mediate the bidirectional communication between the oocyte and its supporting granulosa cells [16-19]. This oocyte-granulosa crosstalk controls the quiescence, activation, and maturation of the primordial follicular pool to regulate both the quantity and quality of the ovarian reserve [20, 21]. Variation or perturbation to these delicate developmental processes, via immune activation for example, can potentially lead to sustained changes in ovarian development, and overall reproductive health [6, 22].

Immune activation is a common perinatal environmental stressor that is modelled experimentally using gram negative bacterial mimetic, lipopolysaccharide (LPS). Lipopolysaccharide provokes an innate immune response by binding to toll like receptor-4 (TLR4). This instigates a proinflammatory cascade via activation of nuclear factor kappa beta (NFkB) and map kinase (MAPK) pathways, and the subsequent secretion of proinflammatory cytokines interleukin (IL) 6, IL1beta (B), tumour necrosis factor alpha (TNFa), C-reactive protein (CRP) and interferon gamma (IFNy) from activated macrophages and immune cells [23, 24]. Animal models of neonatal immune stress via LPS exposure have demonstrated a broad range of long term physiological and behavioural alterations, including neuroendocrine dysfunction, brain morphological alterations, and innate immune system dysfunction [25-28].

The ovary expresses innate immune cells including monocytes, macrophages and adipocytes [29]. Additionally, cytokines and their receptors, and TLRs are locally expressed in ovarian cells and participate in immune functioning essential to ovarian processes [30, 31]. Importantly, LPS exposure has been demonstrated to detrimentally affect female reproductive outcomes [32, 33] including; premature puberty and senescence onset, downregulation of hypothalamic-pituitary-gonadal (HPG) hormone expression, and impairments in mating and maternal behaviours [8, 11, 26, 34, 35]. Moreover, neonatal and adulthood LPS exposure has been demonstrated to lead to in vitro follicular atresia, reduced prepubertal ovarian follicle reserve, and upregulated ovarian TLR4 expression [36-38]. Taken together, these findings suggest that early life LPS exposure produces sustained detrimental effects on ovarian functioning.

To date, little research has focused on the acute in vivo impact of early life immune activation on ovarian morphology and inflammation. Previous studies from our laboratory indicate ovarian inflammatory pathway activation on PND 7 from LPS administration at PNDs 3 and 5 [37], but none to our knowledge have examined the immediate effect of this LPS exposure. Considering the importance of immune involvement for ovarian development and continuing reproductive health [29], the current study aims to examine the acute inflammatory mediators activated by neonatal administration of LPS and associated growth and transcription factors that may underpin the sustained ovarian morphological and behavioural reproductive alterations seen previously in our laboratory with this model, including the early onset of reproductive senescence in female rats, indicated by premature cessation of oestrus cycling [11]. Given that our PND 3 and 5 model of neonatal immune activation falls

within the critical period of ovarian development and sensitivity to immune stress for the rodent ovary [35], we propose that LPS exposure may directly perturb the critical, gonadotropin-independent final stages of neonatal primordial folliculogenesis via excessive immune stimulation occurring both at a systematic and local level.

Methods

Animals and neonatal immune challenge

All animal experimental procedures were undertaken with the approval of the University of Newcastle Animal Care and Ethics Committee (ACEC, A-2012-2813). Twenty one experimentally naïve female Wistar rats were obtained from the University of Newcastle animal house and mated with proven male studs in the Laboratory of Neuroimmunology Vivarium. This resulted in 18 litters and a total of 62 female pups used for this study. Animals were maintained under normal housing conditions at 21-22°C on a 12 hour light/dark cycle (0600-1800) with food available ad libitum. As previously described [11, 26-28, 37, 39], at birth (postnatal day (PND) 1) whole litters were randomly allocated to treatment conditions, either LPS (derived from 10 litters) or saline (derived from 8 litters). Whole litters were exposed to LPS (Salmonella enterica, serotype Enteritidis: Sigma-Aldrich Chemical Co., USA in sterile pyrogen-free Saline, 0.05mg/kg) or Saline (equivolume; Livingstone International, Australia) on PND 3 and again on 5. This low dose and timing of LPS administration has been demonstrated in our laboratory and others to elicit a rapid, sustained and controlled immune and endocrine response during a critical developmental period, without inducing mortality seen at higher doses [26, 28, 37, 40-43]. Briefly, pups were temporarily removed from the home cage and transferred

to an incubator to maintain body temperature, weighed, administered with an intraperitoneal injection of LPS or vehicle, and then returned to the home cage/dam.

Blood and tissue collection

Female pups were euthanized by rapid decapitation. A subset of female animals were culled on PND 3 for analysis of ovarian morphology (*n* = 6 per group derived from 3 litters per group). The remaining females were euthanized on PND 5, at 2 hrs following the last neonatal injection, as this has been demonstrated to be the optimal time point for peripheral, central and genetic cytokine expression following LPS [44, 45]. PND 5 trunk blood was collected into EDTA coated tubes and centrifuged for 20 min at 1000g. Plasma was collected and stored at -20°C for assessment of plasma cytokine levels. Ovaries were dissected with fine tip forceps in 4°C sterile phosphate buffered saline (PBS, Sigma) under a dissection microscope, where all surrounding tissue was removed. One ovary from a subset of animals was randomly chosen and placed in Bouins fixative for histological examination (PND 5; *n* = 6 LPS, 6 Saline, derived from 3-4 litters per group). Remaining ovaries were snap-frozen on dry ice, and stored at -80°C. All remaining tissue was randomly pooled within treatment conditions to create sufficient tissue for biological samples and allocated to either qRT-PCR or proteomic analysis. Male and female animals remaining in litters were allocated to other experiments.

Blood analysis for peripheral inflammatory cytokines.

PND 5 plasma was analysed by ELISA according to the manufacturer's instructions for proinflammatory markers IL6 (Abcam, ab119548 rat ELISA kit, minimum detection rate 12pg/mL, intra- and inter-assay variability <5% and <10% respectively), TNFa (R&D Systems Rat TNF alpha Quantikine ELISA Kit, RTA00, minimum detection rate <5 pg/mL, intra- and inter-assay variability 2.1 - 5.1% and 8.8 - 9.7% respectively) and CRP (Abcam, ab108827, minimum detection rate 0.7ng/mL, intra- and inter-assay variability 3.8% and 9.6% respectively). Each ELISA contained biological samples from at least three different litters per treatment group, with n = 6-12 per group. All samples were assayed in duplicate.

Histological evaluation of ovarian follicles

Ovaries were fixed in Bouins fixative (Sigma Aldrich, castle Hill, Australia) solution for 4h, then washed four times in 70% ethanol, dehydrated, embedded in paraffin and sectioned at 4um. Every 4^{th} slide was stained with hematoxylin and eosin (H&E) for quantification of ovarian follicles, resulting in approximately 8-10 H&E slides per rat neonatal ovary [12, 37]. An experimenter blind to experimental groups examined the samples, and only follicles with a visible oocyte were counted. Primordial, activated primordial, and primary follicles only were classified on H&E sections as follows [see 12]: (1) *Primordial follicle*: an oocyte surrounded by one layer of flattened cuboidal granulosa cells (2) *Activated primordial follicle*: a maturing oocyte surrounded by both flattened granulosa and one or more cuboidal granulosa cells in a single layer (3) *Primary follicles*: an oocyte surrounded by 4 or more cuboidal granulosa cells in a single

layer. Total counts were carried out on the first and third section of every H&E stained slide, resulting in the quantification of all visible follicles [46].

Proteomic identification in ovarian tissue

Post-natal day 5 ovarian protein (10-20 ug) was submitted to the Australian Proteome Analysis Facility (APAF) for proteomic analysis. Prior to submission, protein was extracted from each sample containing 15-20 pooled neonatal ovaries using a modified sodium dodecyl sulfate (SDS) extraction method. Briefly, neonatal ovaries were manually homogenised in extraction buffer (0.375M Tris, ph6.8, 2ml 10%SDS, 3ml MQ H_2O, 1g sucrose), heated at 100°C for 5 min and centrifuged at 13000rpm. Supernatant was removed, then stored and shipped at -80°C. For proteomic analysis, excised gel bands were resized, destained, dried then digested with trypsin in ammonium bicarbonate (pH 8) overnight. Supernatant from gel was made up to 40 uL in ESI loading buffer then was injected onto a peptide trap (Michrome peptide Captrap) for preconcentration and desalted with 0.1% formic acid, 2% ACN, at 8uL/min. The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using a linear solvent gradient, with steps, from H2O:CH3CN (100:0, + 0.1% formic acid) to H2O:CH3CN (10:90, + 0.1% formic acid) at 500nL/min over an 80 min period. The LC eluent was subject to positive ion nanoflow electrospray MS analysis on QSTAR which was operated in an information dependant acquisition mode (IDA). In IDA mode a TOFMS (time of flight mass spectrometry) survey scan was acquired (m/z 400-1600, 0.5s), with the three largest multiply charged ions (counts >25) in the survey scan

sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 2 s (m/z 100-1600). The data were processed using the database search program, Mascot (Matrix Science Ltd, London UK) with peaklists searched against Rattus in the SwissProt database [47]. High scores in the database search indicate a likely match that was confirmed or qualified by operator inspection. Search results were generated with a significance threshold of $p < 0.05$ with an ion score cut-off of 25 for all samples. This work was undertaken at APAF, the infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS). LPS and saline groups were compared and Ingenuity Pathway Analysis (IPA: Ingenuity Systems, Redwood City, CA) software was used to identify top canonical signalling protein pathways and upstream regulators affected by neonatal treatment.

RNA extraction, Reverse Transcription and Real-Time Quantitative (qRT)-PCR

In order to isolate sufficient quantity and quality mRNA from whole PND 5 ovaries, 6-8 ovaries from the same treatment group were randomly pooled within treatment groups to create biological replicates. Total RNA was isolated from ovaries using a modified acid guanidinium thiocyanate-phenol-chloroform protocol, followed by an isopropanol precipitation as previously described [48, 49] and DNase treated prior to reverse transcription for the removal of genomic DNA. Reverse transcription was performed as outlined in Sobinoff, et al. [48] with 2 ug of total isolated RNA, 500ng oligo(dT), 15 ug of primer (FWD and REV), 40ug of RNasin, 0.5mM dNTPs, and 20 ug of M-MLV-Reverse Transcriptase (Promega; Madison, WI, USA). Reverse transcription reactions were verified by Actin beta *(Actb)* or Cyclophilin qPCR using cDNA amplified with GoTaq

Flexi (Promega). Quantitative RT-PCR was performed in 20ul reactions using SYBR Green GoTaq qPCR master mix (Promega) according to manufacturer's instructions on a LightCycler 96 SW 1.0 (Roche, Castle Hill, NSW, Australia) for transcription factor Forkhead box O3a (*Foxo3a*) and growth differentiation factor 9 (*Gdf9*), as well as inflammatory markers; *Tnfa*, mitogen activated protein kinase 8/Jun N-terminal kinase (*Mapk8/Jnk1*), protein kinase C beta (*Prkcb*) and *Tlr4*. These markers are associated with both LPS activated inflammatory pathways and are essential to steroidogenesis during this critical time-point in gonadotropin-independent ovarian development, as well as continued ovarian-immune functioning throughout the lifespan [18, 50-60]. Gene and protein markers assessed were chosen to reflect the nature and timing of our early life immune stress exposure model, based on previous research from our laboratory [37] and in line with broad proteomic identification of factors in the current study associated with ovarian cell proliferation, migration, apoptosis, and LPS induced inflammation (see supplementary data table S5). Each sample was accompanied by a RT-negative replicate as a negative control. Quantitative RT-PCR data were normalised to the housekeeping control gene Cyclophilin as per Sutherland, et al. [49] and analysed using the comparative C_T method equation $2^{-\Delta\Delta C(t)}$ (where C(t) is the threshold cycle at which fluorescence is first detected as statistically significant above background) and presented as a fold increase relative to the saline control group [61]. Experiments were replicated a minimum of 3 times prior to statistical analysis, with all PCR performed on at least 3 separate tissue isolations/biological replicates [as per 62]. Primer sequences are supplied (table 1) and were optimised by qPCR both here and previously [37].

Immunohistochemistry

Immunohistochemistry was used to localise TNFa protein and DNA damage via Phosphorylated histone gamma H2AX (yH2AX) expression. Caspase 3 (CASP3) and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining were used to quantify apoptosis. Toll like-receptor 4 localization was assessed to confirm LPS activation via TLR4 binding in the neonatal ovary. TNFa was localised in PND 5 ovarian tissue using a Vector DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA, USA) following manufacturer's instructions. Slides were deparaffinised in xylene and rehydrated in ethanol washes. Antigen retrieval was carried out in preheated Na citrate buffer (10mM, pH6), microwaved for 12 min. Endogenous peroxidase quenching was performed (0.3%) for 20 min; slides were rinsed in PBS-TX and blocked in 3% BSA in PBS for 1 hour. Slides were incubated with primary antibody overnight at 4°C (anti-TNFa, Abcam ab6671, 1:200 dilution), then rinsed with PBS-TX and incubated with biotinylated secondary antibody (rabbit IgG; Abcam ab191866, 1:500) for 30 min. Sides were rinsed, incubated with Vectorstain ABC prepared to manufactures instructions for 30 min at room temperature, then incubated with DAB for 2 min, counterstained with Carezzis blue for 2 min, rinsed in bluing solution, dehydrated in ethanol and xylene, then mounted and viewed using an Axio imager A1 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). Images were taken using an Olympus DP70 microscope camera (Olympus America, Centre Valley, PA, USA). For TLR4, yH2AX, immunohistochemistry was carried out following the dewaxing, rehydrating and antigen retrieval previously mentioned. Cooled slides were blocked in 3% BSA/Tris-buffered Saline (TBS) for 1 hour at room temperature. Sections were incubated

overnight at 4°C with anti-TLR4 (1:100; Santa Cruz, sc-16240), anti-yH2AX (1:200; Abcam ab26350) and anti-CASP-3 (cleaved form, ~17kDa, 1:100; Abcam, ab13847). Slides were washed in TBX (0.1% Triton X-100) and incubated with appropriate fluorescent conjugated secondary antibodies (Alexa Fluor 594 goat anti-rabbit/goat anti-mouse IgG, 1:200, Abcam; ab150080, ab150120 respectively). TUNEL was performed using an ApopTag Fluorescein in situ Apoptosis Detection Kit (Millipore, S7110) according to the manufacturer's instructions. Sections for immunofluorescence were counterstained with either YOYO-1 nuclear stain (green) or DAPI (blue), mounted with Mowiol and viewed as described above. Positive controls included treated mouse and rat reproductive tissues and spleen for antibody specificity and DNase treated tissue where appropriate, and no primary antibody/TdT enzyme negative controls on target tissue (see supplementary tables S1 for antibody information and S6 for IHC control imaging). TUNEL positive cells were quantified by an experimenter blind to treatment conditions. Caspase-3 corrected total cell florescence (CTCF) was measured in Image J (National Institutes of Health, MD, USA) and calculated using the formula CTCF = integrated density – (area of selected section x mean fluorescence of background readings) [63].

Statistical analysis

Data were analysed using IBM SPSS statistics (Version 24, IBM Australia) with a two-way ANCOVA design, repeated measures ANOVA, and student independent t-test where appropriate, with pairwise comparisons between treatment groups carried out using the Bonferroni correction. Where covariates including litter size, body weight, and

male-to-female ratio did not significantly impact dependent variables, they were removed from the analysis to maximise statistical power. Statistical assumption violations were corrected by a log10 transformation (IL6 only). Data are presented here as the mean + standard error of the mean (SEM). Significance was assumed at $p \leq 0.05$.

Results

Neonatal weight gain

No significant weight difference was observed between treatment groups on PND 3 or PND 5, however, LPS females gained significantly less weight between PND 3 and 5 compared to controls (Neonatal Treatment x Age: $F_{(1, 57)} = 42.02$, $p \leq .0001$; pairwise contrast: $t(43) = 4.5$, $p < .0001$; Fig 1A). An expected significant main effect of age ($F_{(1, 57)} = 105.49$, $p < .0001$) was also observed, with PND 5 animals weighing more than PND 3 animals.

Impact of LPS on peripheral inflammatory markers on PND 5

As anticipated, circulating CRP, IL6 and TNFa was significantly upregulated in LPS treated animals compared to controls ($F_{(1, 17) =} 11.782$, $p = .003$ (Fig 1B), $F_{(1, 15)} = 10.47$, $p = .006$ (Fig 1C), $F_{(1, 24)} = 4.71$, $p = .04$ (Fig 1D), respectively).

Impact of LPS on early ovarian follicle pool

On PND 5, LPS treated animals had significantly reduced primordial follicles compared to saline controls (t (10) = 4.02 $p = .002$, Fig 2A; Neonatal Treatment effect: $F_{(1, 20)} =$

18.78, $p < .001$; Neonatal Treatment x Age effect: $F_{(1, 20)} = 10.90$, $p = .004$). Neonatal LPS exposure altered the quantity of activated primordial follicles, with *reduced* numbers of follicles in LPS treated rats compared to saline controls on PND 3 (t (10) = 2.39, $p = .03$, Fig 2B), but significantly *increased* activated primordial follicles on PND 5 (t (10) = 2.31, $p = .044$); Age x Treatment effect: $F_{(1, 20)} = 10.97$, $p = .003$; Fig 2 B). There were no significant differences in primary follicle numbers on either treatment day (Fig 2C). As expected, a significant effect of age was seen on all follicle types (primordial: $F_{(1, 20)} = 104.4$; activated primordial $F_{(1, 20)} = 13.48$; primary; $F_{(1, 20)} = 26.47$, $p < .005$ for all), with PND 5 ovaries containing more follicles overall compared to PND 3.

Impact of LPS on ovarian proteome

There was significant expression of 598 proteins in the ovaries of PND 5 LPS females ($p \leq .05$). Protein expression was compared to saline control levels, resulting in 29 proteins differentially expressed in LPS treated animals. Functional analysis of these identified several molecular networks, canonical pathways and cellular functions implicated in acute phase response signalling and innate immune responses, amino acid and lipid metabolism, molecular transport, and cellular movement, signalling, assembly and survival (Fig 3; A, B & supplementary tables S2 - S5).

Ovarian qRT-PCR

PND 5 ovaries were probed for mRNA expression of proinflammatory markers and growth and transcription factors. Fold-change mRNA expression of *Gdf9* was

significantly upregulated in the ovaries of PND 5 LPS treated females (t (4) = 8.05), compared to saline controls, $p \leq .001$ (Fig 4A). There were no significant changes in expression of *Foxo3a* (Fig 4 B). We observed a trend towards downregulated *Tlr4* mRNA expression in LPS treated animals ($p = .061$) (Fig 4C). Expression of *Tnfa* and *Mapk8/Jnk1* were significantly upregulated in LPS treated animals on PND 5, (t (4) = 3.54 and t (4) = 0.14, respectively; both $p \leq .05$), (Fig 4; D, E). LPS females displayed a non-significant increase in *Prkcb* mRNA expression ($p = .170$, Fig 4F).

Ovarian protein localization and quantification

Immunohistochemical processing was carried out on the PND 5 ovaries of LPS and saline treated females to detect the localized expression of TLR4 and TNFa protein, yH2AX as a marker of double stranded DNA damage, and cleaved CASP-3 and TUNEL for assessment of apoptosis. Gamma H2AX was detected in oocytes of both LPS and saline animals (Fig 5; A, B). Toll-like receptor 4 immunolabelling was detected surrounding the oocyte (Fig 5; C, D). Tumour necrosis factor alpha expression was detected both in the granulosa cells and oocytes in both treated and control samples (Fig 5; E, F). Caspase-3 quantification in the ovaries of LPS treated animals demonstrated a 2.3 x fold change increase in CTCF compared to saline treated controls. However, this increase was not statistically significant (t (4) = 2.291, $p = .084$ (Fig 6; A – C). LPS treated animals demonstrated a significantly greater number of TUNEL positive ovarian cells (t (4) = 5.191, $p = .007$, particularly in oocytes (Fig 6; D – F).

Discussion

Early life is a critical period for fundamental ovarian development and associated neuroendocrine and immune system maturation. Disruption of developmental trajectories via immune activation during this sensitive period may have persisting detrimental effects. Here, we demonstrate in female neonatal rodents that a low dose of LPS in the first week of life has an acute effect on the neonatal ovary during the final stages of follicular pool formation in vivo. Lipopolysaccharide administration on PND 3 and 5 upregulated circulating inflammatory mediators, altered early follicle populations, and had an immediate effect on ovarian immune status on PND 5. This study is one of the first to show the immediate effect of perinatal immune stress on early ovarian follicle populations and associated ovarian transcriptome.

Neonatal LPS exposure resulted in reduced weight gain between PND 3 and PND 5, consistent with our previous findings [11, 27, 28]. As expected, administration of LPS on PND 3 and 5 caused significant increases in circulating acute phase protein CRP, IL6 and TNFa. These results confirm the efficacy of LPS treatment in what is considered a hypo-responsive period for immune responses and stress in the neonatal rodent [64, 65]. Peripheral increases in proinflammatory cytokines may stimulate and exacerbate normal inflammatory mediator levels within the ovary at this critical time, regardless of ovarian immune privilege.

Neonatal LPS exposure altered early follicle populations in the PND 5 ovary, leading to a significant decrease in primordial follicle numbers and a significantly greater number of activated primordial follicles. This suggests that early life immune activation may prematurely activate quiescent follicles, leading to follicle depletion. Previous

findings from our laboratory demonstrated a depleted PND 14 primordial follicle pool [37], as well as advanced senescence at 1 year of age [11], indicating that the morphological changes seen here are sustained through to prepubescence and may contribute to an early fertility decline. Premature follicle diminishment reduces the number of viable follicles for later ovulation, in turn affecting the quality of the dominate follicle. The untimely depletion of primordial follicle pool seen here may be occurring via intra-ovarian autocrine and paracrine immune signalling [66] or via gap junctions operating within the oocyte-granulosa cell complex [67], stimulated by excessive immune perturbation. Rapid follicle formation, proliferation, maturation and atresia is typical in the PND 2 to 5 female rodent, where oogonia migration is occurring [15]. LPS administration may exacerbate these typical ovarian processes, particularly as LPS stimulation has been demonstrated to cause oocyte and granulosa cell apoptosis in fully developed ovaries [36, 68]. This is substantiated in the current study by the localization of yH2AX in the oocyte complex, indicative of rapid DNA damage [69], and the significant increase in TUNEL positive oocytes in LPS treated animals, demonstrating a combined effect of PND 3 and PND 5 LPS injections. Primary follicle populations did not differ, suggesting that primordial follicles may have already undergone apoptosis post-activation, and that the primordial follicle population may have a heightened vulnerability to the effects of LPS. Likewise, Bromfield and Sheldon [36] demonstrated that the primordial follicle pool is particularly sensitive to LPS driven apoptosis, whereas larger follicles display some resilience.

Using mass spectrometry, we identified 29 proteins differentially expressed in the PND 5 ovaries of LPS treated animals. Pathway analyses indicated that these proteins

were associated with acute-phase response signalling, liver X receptor/retinoic X receptor (LXR/RXR) activation, mechanism of viral exit, and glycogen degradation signalling (see Fig 3 & supplementary tables S2 - S5). This indicates transcription of acute phase mediators within the PND 5 ovary, confirmed here with qRT-PCR. These results are congruent with our previous microarray findings demonstrating LPS stimulated upregulation of immune pathways in PND 7 ovaries [37]. Activation of the LXR/RXR pathway is of novel interest, as LXR/RXRs are involved in the macrophage response to TLR4 activation and are expressed in the ovary [70, 71], with LXR null mice displaying subfertility and oocyte meiotic incompetence [72]. Interestingly, LXR/RXR activation inhibits inflammatory signalling [73], hence activation of LXR/RXR seen here may serve to protect the developing ovary from excessive inflammation. Further investigation is needed as this may be a novel pathway to examine in the early life immune stress model, particularly considering the association between early life stress, fertility dysfunction, and metabolic and inflammatory diseases.

In the current study, LPS administration significantly upregulated the mRNA expression of inflammatory mediators MAPK8/JNK1 and TNFa in the ovaries of LPS treated animals on PND 5. MAPK8/JNK1 signalling is a major component in acute phase responses, cell survival and apoptosis [60] and both LPS and proinflammatory cytokines, particularly TNFa, activate the MAPK8/JNK1 pathway [74, 75]. The MAPK8/JNK1 pathway is also implicated in the activation and maturation of early follicles [60, 76]. Recent evidence indicates MAPK8/JNK signalling contributes to follicle activation via mTOR signalling, which may merit further investigation within the current model [77]. The significantly increased expression seen here may indicate a

downstream acute phase response is activated within the ovary, with immune mechanisms contributing to early follicle depletion. Concomitant to MAPK8/JNK1 stimulation, is the activation of other inflammatory pathways and mediators including NFKB, PI3K/AKT, PKCB, IL1B, IL6, nitric oxide synthase-1 (NOS2) and cyclooxygenase-2 (COX2) [78], which are all associated with both normal and pathological ovarian function [66, 79-82], and early life activation of MAPK pathways may lead to long term functional differences with these mediators.

It is known that TNFa activates and exacerbates the LPS-driven immune and stress responses, and plays a fundamental role in the immature ovary, facilitating normal oocyte atresia and follicular assembly to define the size of the primordial follicle pool [58, 83]. As TNFa alone can impair ovarian functions and override factors that inhibit follicle activation [59, 84], these higher gene expression levels of *Tnfa* seen here may be a driving factor increasing primordial follicle activation in LPS treated animals, leading to superfluous and premature depletion. Tumour necrosis factor alpha protein was localized to granulosa cells, oocytes, and the surrounding complex in both groups, indicating that expression may be facilitating bidirectional crosstalk between the oocyte-granulosa matrixes. The strong trend for *Tlr4* downregulation seen here is most probably due to a habituation effect of the temporal proximity of dual LPS administration and the time point at which tissue was taken. These findings were contrary to those hypothesised, as we previous demonstrated a significant upregulation of *Tlr4* gene expression in the PND 7 ovary, however TLR4 staining here was localised to the oocyte cytoplasm as previously demonstrated [37]. The current and previous findings suggesting that the ovary may adapt to the level of LPS signalling and display altered

TLR4 expression as a way to manage a predicted high-immune-stress or bacterial-rich environment. Permanent alterations in TLR4 expression may be detrimental, particularly considering that TLR4 overexpression is involved in the growth and survival of ovarian cancer cells [85] and poor ovarian response (POR) [86], and TLR4 under expression is implicated in polycystic ovarian syndrome (PCOS) and endometriosis [87, 88]. Protein Kinase C beta aids the regulation of early follicular proliferation, survival and activation [89] and although upregulation here is non-significant, it may be contributing to the perpetuation of cytokine secretion [90].

Growth factor GDF9 and transcription factor FOXO3A are implicated in early follicle primordial growth, maturation and apoptosis. The significantly increased *Gdf9* mRNA expression demonstrated here may indicate that immune activation is prematurely instigating the maturation signalling between the oocyte and its granulosa cells, particularly as GDF9 stimulates small follicle proliferation of granulosa cells in rats in vivo [91, 92] and promotes growth of human ovarian follicles in vitro [93]. Abnormal expression of GDF9 and *Gdf9* mutations of are apparent in women with PCOS and POF [53, 94]. Additionally, the *Gdf9* increases seen here may also be an attempt to downregulate inflammation, as *Gdf9* activation stimulates transforming growth factor (TGF)-beta-like SMAD2/3 intracellular pathways [95] which have been shown to inhibit immune cells, including macrophage activation, with LPS exposure [96]. As macrophages that express TLR4 are present in the ovary, the increase in *Gdf9* expression in LPS challenged animals may indicate a protective mechanism undertaken by the ovary in order to self-modulate inflammatory signalling, with activation and atresia of some early follicles being compensation for overall ovarian reserve defence.

Neonatal immune insults may not only impact the quantity, but also the long term quality of the remaining follicular pool. As GDF9 and associated TGF-b superfamily members are modulators of sex hormone sensitivity, aberrant GDF9 signalling during ovarian development may have long term effects on follicle stimulating hormone (FSH) and luteinising hormone (LH) receptor densities and sensitivities, affecting post-pubertal ovarian processes [97, 98]. Forkhead box-O3a has been suggested as a key mediator of naked oocyte and primordial follicle apoptosis within the neonatal rat ovary [56]. As FOXO is negatively regulated by PKB/AKT/PI3K and MAPK8/JNK1 activation [55, 99], this may be contributing to our unexpected non-significant findings as previous findings with this LPS model indicate significant upregulation of *Prkb/Akt/Pi3k* pathways in PND 7 ovaries [37]. The non-significant difference in *Foxo3a* at this current time-point indicates that analysis at an alternate time point is needed to capture *Foxo3a* translocation, transcription and apoptosis induction. Forkhead box O3-null mice demonstrate normal follicle assembly, followed by global activation and early depletion of the primordial follicle pool, leading to POF and infertility [100]. This implicates FOXO3A in the maintenance of quiescent follicles, as transgenic models of constitutively active *Foxo3a* expression demonstrate suppression of follicular maturation and infertility [101], meriting future investigation in this model. Future studies are necessary to examine FOXO3A and additional growth/transcription factor gene and protein expression in the neonatal LPS model, particularly given the links between FOXO3A and LPS-induced innate inflammatory pathway upregulation, ovarian follicular development, and studies demonstrating LPS inactivating FOXO3A in other tissues [102-104].

Our current findings indicate that LPS exposure activates and depletes the early primordial follicle pool. This may be occurring via immune pathways to affect transcription and growth factor-mediated early follicular control, particularly considering the shared, complex immune-regulatory properties and capabilities of TNFa, MAPK8/JNK1, and GDF9. These mediators influence early follicle formation, development, and maintenance during a critical period, where follicle dynamics are both intricate and elusive in nature. The development of the finite ovarian follicular pool is dependent on homeostatic processes, which if disrupted, may lead to sustained alterations to ovarian physiology. Premature loss of the ovarian reserve not only has a detrimental effect on the female reproductive lifespan, but is associated with a myriad of heath complications due to deficiency of ovarian produced oestrogen, including osteoporosis, cardiovascular disease, autoimmunity and psychological disorders. Early life stress is already a well-established risk factor for morbidity and mortality from a range metabolic, immune and neuroendocrine disorders [105], and is known to lead to a physiological vulnerability to stressors in later life [43, 106]. The pathogenesis of idiopathic reproductive disorders may have developmental origins, as well as be intensified by the additional stress these disorders exert. The current study provides further insight into the link between early life immune disturbances and ovarian development. A number of female reproductive disorders associated with skewed inflammatory profiles such as; PCOS, endometriosis, and POF are increasingly presenting in a younger female demographic, often with no apparent origin. Examining stressful events in the early life environment can therefore provide valuable insight into the pathogenesis and progression of reproductive disorders, as well as aid in the

understanding of mechanisms regulating the formation and longevity of the ovarian reserve.

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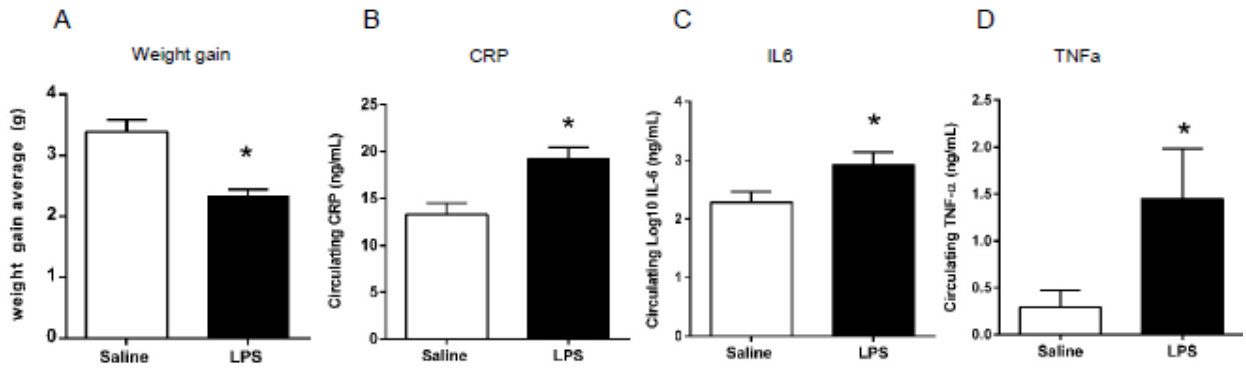

Figure 1. Weight gain between PND 3 and PND 5 and circulating proinflammatory cytokines. A) Neonatal females treated with LPS (*n* = 29) gained significantly less weight between treatment days compared to saline treated females (*n* = 31), mean difference in weight gain between groups represented in grams. LPS treated animals displayed significantly increased plasma levels of B) CRP, C) IL6, and D) TNFa. White bars represent saline controls, filled bars represent LPS treated animals. * indicates $p < .05$.

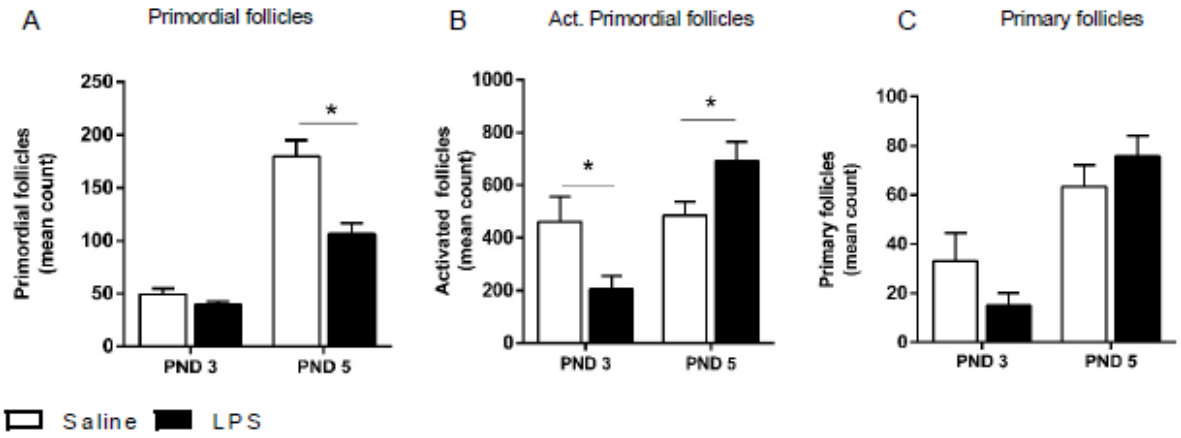

Figure 2. PND 3 and 5 mean ovarian follicle counts. Mean counts of early follicle populations A) primordial follicles on PND 3 and PND 5; B) Activated primordial follicles on PND 3 and PND 5; C) Primary follicles on PND 3 and PND 5. Mean ovarian follicle count +SEM is graphed. White bars represent saline treated controls, filled bars represent LPS treated animals, * indicates $p < .05$.

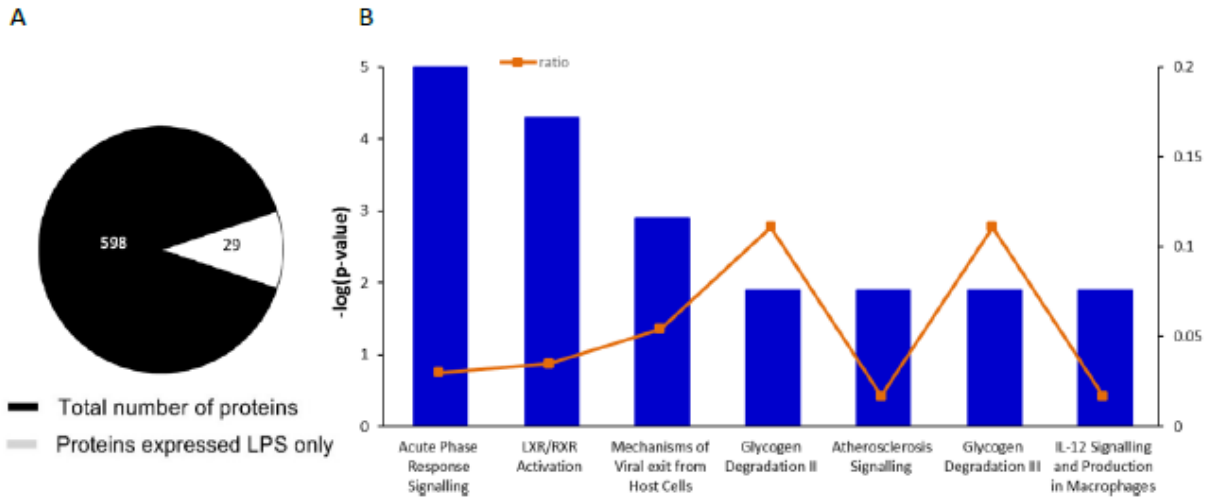

Figure 3. Mass Spectrometry and Top Canonical Pathways significantly upregulated by neonatal LPS treatment on PND 5, as identified by Ingenuity Pathway Analysis (IPA) A) Representation of mass spectrometry information obtained from PND 5 LPS-treated ovaries. 29 proteins were significantly differentially expressed in the ovaries of LPS-treated females out of the total number of proteins significantly expressed, compared to controls. B) Blue bars (column graph, x-axis) represents the significance of associated upregulated expressed proteins and the canonical pathway assessed using a right-tailed Fisher's exact test to calculate p-values determining the probability that the association is explained by chance alone. Ratio score (line graph, z-axis) indicates the proportion of coverage from a pathway related to total number of molecules, subject to pathway size bias.

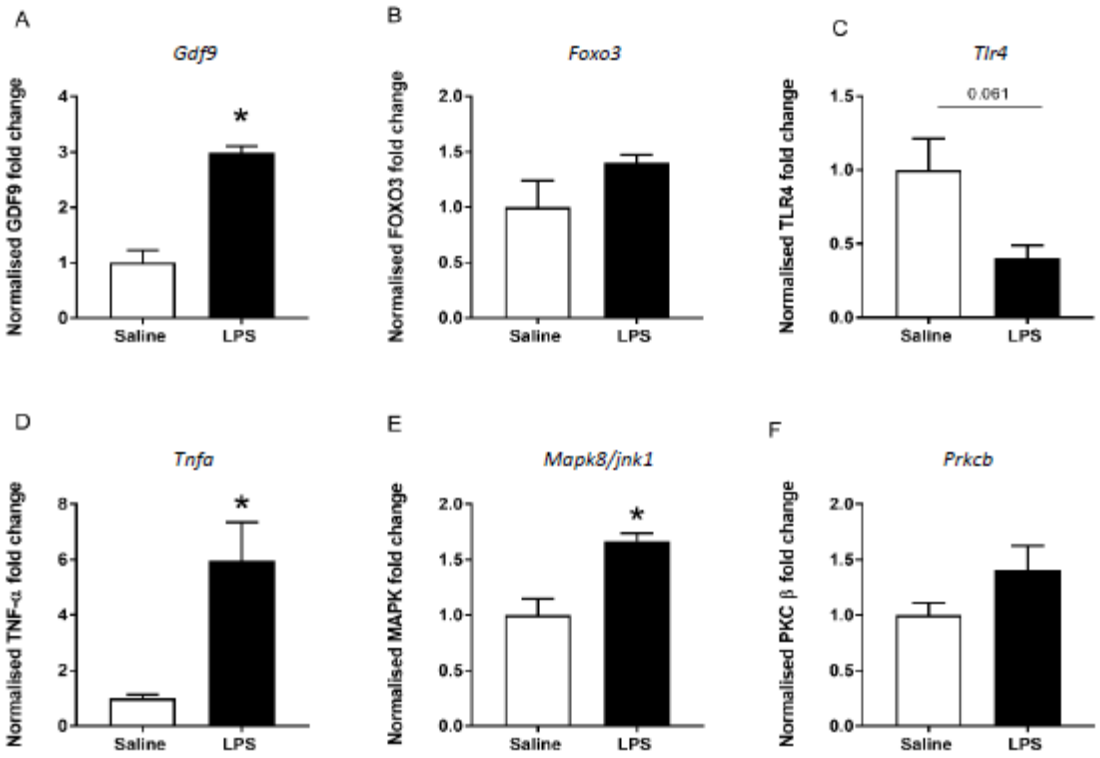

Figure 4. QRT-PCR analysis of growth and transcription factors, and inflammatory pathways associated with initial folliculogenesis. Fold change mRNA expression of ovarian growth factor *Gdf9* (A) and transcription *Foxo3*a (B). Fold change mRNA expression of inflammatory mediators associated with ovarian follicular development and function; *Tlr4* (C), *Tnfa* (D), *Mapk8/Jnk1* (E), and *Prkcb* (F). White bars represent saline controls, filled bars represent LPS treated animals. * indicates $p < .05$.

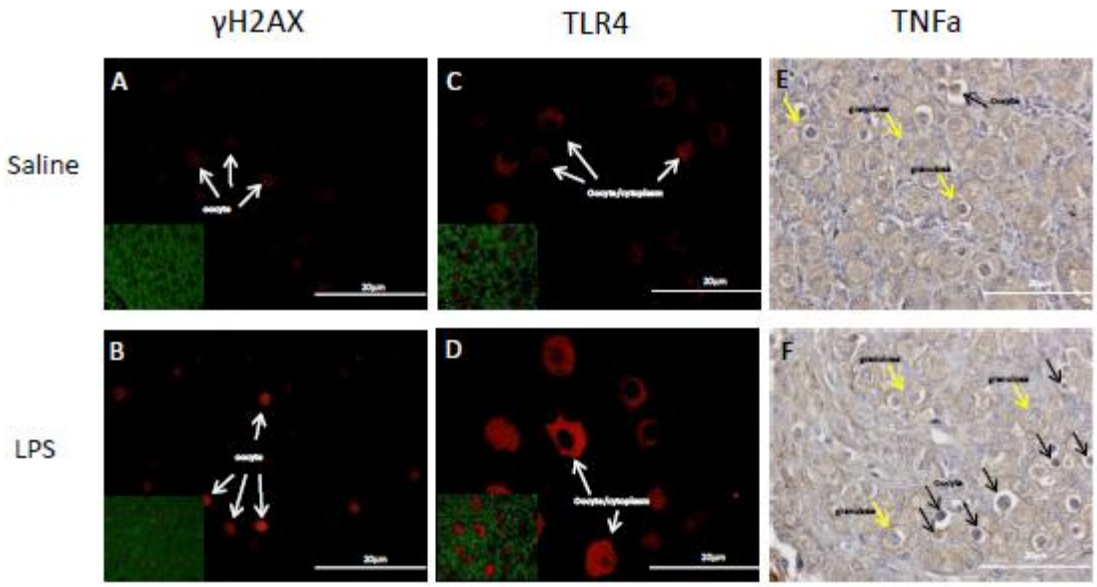

Figure 5. Observational fluorescent and Immunohistochemical protein localization of γH2AX, TLR4 TNFa. (A-B) Representative γH2AX immunolabelling detected in oocytes in both treatment groups; (C-D) TLR4 immunolabelling was expressed in the oocytes and oocyte cytoplasm in both treatment groups; (E-F) TNFa expression was localized to the oocyte and surrounding granulosa cells in both LPS and saline. Fluorescent green (YOYO) represents nuclear staining, fluorescent red staining represents specific staining for protein of interest (A-D). White arrow = area of interest. DAB TNFa staining is indicated in brown, counterstained with Carezzis blue (E-F). Yellow arrow = granulosa, black arrow = oocyte.

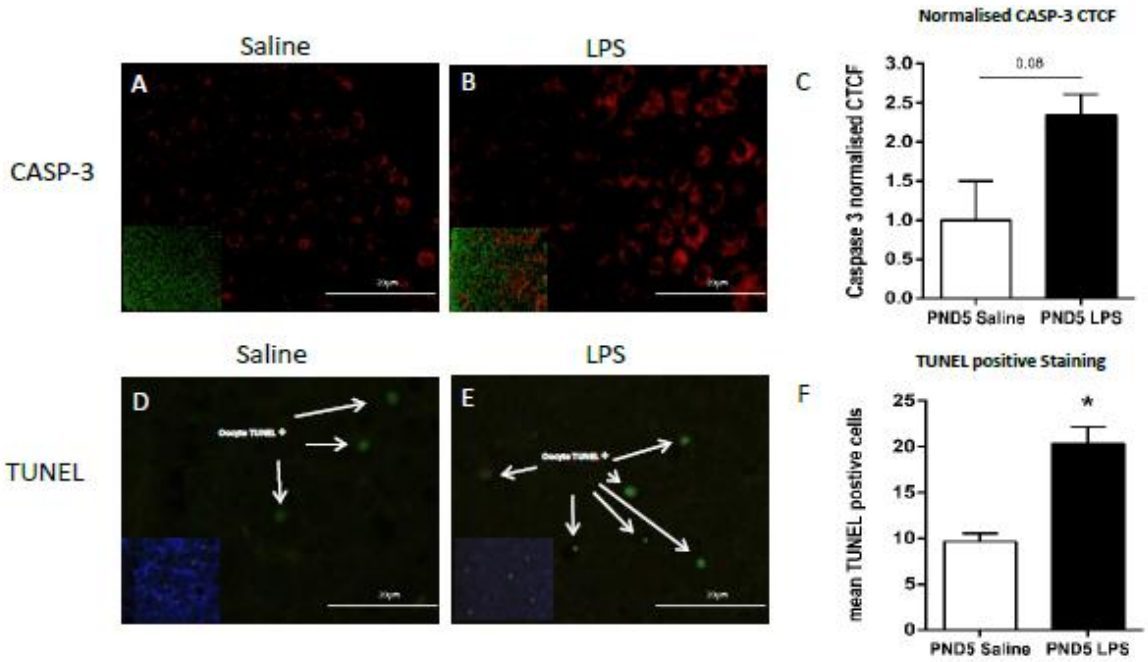

Figure 6. Quantification of cleaved CASP-3 and TUNEL positive cells in PND 5 ovaries. Localisation representative images of cleaved CASP-3 in A) saline treated females and B) LPS treated females. Fluorescent green (YOYO) represents nuclear staining, fluorescent red staining represents CASP-3 staining. C) Quantification of CASP-3 represented as normalised fold change CTCF compared to saline controls +SEM. Localisation representative images of TUNEL positive ovarian cells in D) saline treated females and E) LPS treated females. F) Quantification of TUNEL positive cells in saline and LPS treated groups represented as mean count +SEM. Fluorescent blue (DAPI) represents nuclear staining. White bars represent saline controls, filled bars represent LPS treated animals. * indicates $p < .05$.

Table 1. qRT-PCR primer information. Forward and reverse sequence and efficiency for target genes analysed involved in early ovarian follicular control and development, and inflammation.

Target Gene	Forward	Reverse	efficiency
Mapk8/Jnk1	*CGGAACACCTTGTCCTGAAT*	*GAGTCAGCTGGGAAAAGCAC*	1.94
Prkcb	*ATCAGCCCTACGGGAAGTCT*	*CGTTGTGCTCCATGATTGAC*	1.91
Tlr4	*ACTGGGTGAGAAACGAGCTG*	*CGGCTACTCAGAAACTGCCA*	1.97
Actin B	*TCTGTGTGGATTGGTGGCTCTA*	*CTGCTTGCTGATCCACATCTG*	1.94
Cyclophilin A	*CGTCTCCTTCGAGCTGTTT*	*ACCCTGGCACATGAATCCT*	1.9
Gdf9	*CAACCAGATGACAGGACCC*	*AGAGTGTATAGCAAGACCGAT*	1.83
Foxo3a	*CACAGAACGTTGTTGGTTTG*	*CAGTTTGAGGGTCTGCTTTG*	1.84