CSIRO PUBLISHING

Reproduction, Fertility and Development
https://doi.org/10.1071/RD17241

Effect of lipopolysaccharide on the expression of inflammatory mRNAs and microRNAs in the mouse oviduct

Katheryn L. Cerny[A], *Rosanne A. C. Ribeiro*[A], *Qing Li*[A], *James C. Matthews*[A] *and Phillip J. Bridges*[A,B]

[A]Department of Animal and Food Sciences, University of Kentucky, Lexington, KY 40546, USA.
[B]Corresponding author. Email: phillip.bridges@uky.edu

Abstract. Infection with Gram-negative bacteria is a major cause of aberrant inflammation in the oviduct; consequences can include tubal-based infertility and/or ectopic pregnancy. Understanding the inflammatory response is necessary for the development of novel treatment options that counter inflammation-induced infertility. The aim of the present study was to determine the effect of intraperitoneal (i.p.) administration of *Escherichia coli*-derived lipopolysaccharide (LPS) on the acute expression of inflammatory mRNAs and microRNAs (miRNAs) in the oviduct. On the day of oestrus, 6- to 8-week-old CD1 mice were injected i.p. with 0, 2 or 10 μg LPS in 100 μL phosphate-buffered saline. Mice were killed 24 h later and the oviducts collected for gene expression analyses. The effect of treatment on the expression of mRNAs and miRNAs was evaluated by one-way analysis of variance (ANOVA), with treatment means of differentially expressed ($P < 0.05$) transcripts separated using Scheffé's test. LPS treatment affected 49 of 179 targeted inflammatory mRNAs and 51 of 578 miRNAs ($P < 0.05$). The identity of differentially expressed miRNAs predicted as regulators of chemokine and interleukin ligand mRNAs was then extracted using the microRNA.org database. The results of the present study indicate that systemic treatment with LPS induces a robust inflammatory response in the oviducts of mice, and identify key mRNAs and putative miRNAs modulating this effect.

Additional keywords: cytokine, gene expression, inflammation, nanostring.

Received 13 December 2016, accepted 25 August 2017, published online 26 September 2017

Introduction

Salpingitis or aberrant inflammation of the oviduct is one of the most common forms of pelvic inflammatory disease (PID), and is one of the most important components of the PID spectrum due to its effect on female fertility (for a review, see Sweet 2011). This uncontrolled inflammation results in oviductal epithelial cell death, tubal scarring and eventually occlusion (Punnonen *et al.* 1984; Donnez and Casanas-Roux 1988; Perfettini *et al.* 2000; García-Ulloa and Arrieta 2005; Steffl *et al.* 2008). The incidence of tubal pathology is high (survey data indicate that tubal dysfunction is comparable in aetiology to ovulatory defects or endometriosis as an indication for the treatment of female infertility; Wright *et al.* 2008) and secondary effects, including inflammation-induced tubal ectopic pregnancies, can be life threatening (Shao 2010; Shaw *et al.* 2010; Creanga *et al.* 2011). Therefore, understanding the inflammatory response is necessary for the continued development or refinement of treatment options that specifically target inflammation within this organ.

Inflammation of the oviduct is often a continued immune response to infection with sexually transmitted bacterial pathogens (Mårdh 2004). These pathogens are known to target the oviduct, manipulating signalling pathways in an intricate balance to both exploit and protect the host (Koomey 2001; Zhong 2009). Infection with *Chlamydia trachomatis* is currently at record high levels in the US (Centers for Disease Control and Prevention 2016) and treatment of *Neisseria gonorrhoeae*, the second most reported of these notifiable sexually transmitted diseases, is becoming increasingly plagued by resistance to antibiotic therapy (Deguchi *et al.* 2010). Several mouse models are routinely used to investigate both *Chlamydia*- (Rasmussen *et al.* 1997; Maxion and Kelly 2002; Zhong 2011; Kessler *et al.* 2012; Igietseme *et al.* 2013) and *N. gonorrhoeae*-induced (Feinen and Russell 2012; Hobbs *et al.* 2013; Liu *et al.* 2013) genital infection. The importance of these animal models to the investigation of inflammation in the oviduct is unequivocal; however, the response to infection can be dependent upon bacterial serovar, route of administration, dose and, importantly, the genetic strain of mice used within any particular study (Tuffrey *et al.* 1986; Darville *et al.* 1997, 2001).

To circumvent the potential inconsistencies associated with investigating inflammation of the mouse oviduct using these models, the aim of the present study was to determine the response within the oviduct to a systemic injection of *Escherichia coli*-derived lipopolysaccharide (LPS), an animal model previously characterised to study inflammatory responses of the uterus, including implantation (Deb *et al.* 2004), embryo

Journal compilation © CSIRO 2017

viability (Mayorga *et al.* 2004) and pregnancy loss (Diamond *et al.* 2007; Phillippe *et al.* 2011). Importantly, the endotoxin, the hydrophobic anchor of LPS, is a glucosamine-based phospholipid of the outer membranes of most Gram-negative bacteria (for a review, see Raetz and Whitfield 2002), including *C. trachomatis* and *N. gonorrhoeae.* A targeted nanostring approach was used to determine the effect of treatment with LPS on the expression of inflammatory mRNAs within the oviduct, and a more inclusive microRNA (miRNA)-based nanostring assay was used to identify putative miRNAs that may be inducing post-transcriptional modification of inflammatory gene function. For mRNAs encoding the chemokine and interleukin ligands that were included in the inflammatory mRNA panel, miRNAs that were predicted to target each mRNA and were affected by treatment with LPS were then identified. Overall, the effects of treatment with LPS on the level of expression of approximately 750 mRNAs and/or miRNAs were investigated. Those mRNAs and miRNAs affected by treatment are reported herein; however, the physiological importance of mRNAs and/or miRNAs that did not differ with treatment cannot be discounted.

Materials and methods

Animals and tissue collection

Animal procedures involved in this study were approved by the University of Kentucky Animal Care and Use Committee. Prior to treatment, the progression of normal oestrous cycles was confirmed in 6- to 8-week-old CD1 mice by analysis of vaginal cytology, as described previously (Jeoung and Bridges 2011; Bridges *et al.* 2012). Briefly, vaginal smears were collected daily, at the same time each day, using phosphate-buffered saline (PBS) and a bent, blunted borosilicate glass pipette. Vaginal cytology was evaluated under a Motic AE21 inverted microscope (Motic Instruments), classified according to well-established morphological criteria (Caligioni 2009) and digital images recorded for later reference. On the morning of oestrus, mice ($n = 8$ in each group) were injected intraperitoneally with 0 (control) or 2 or 10 μg LPS from *E. coli* serotype 055:B5 (Sigma-Aldrich) in 100 μL PBS (low- and high-dose groups respectively). The rationale for treatment on the morning of oestrus was that treatment at oestradiol-induced sexual receptivity will induce an inflammatory response at the time of typical exposure of the oviduct to freshly ovulated cumulus–oocyte complexes, associated follicular debris, spermatozoa, seminal fluids and, putatively, any foreign pathogens introduced at mating. To investigate the acute response to LPS-induced inflammation, mice were killed 24 h later and whole oviducts collected, frozen on dry ice and then stored at −80°C (for ∼1 month) before extraction of total RNA.

RNA extraction and analysis

To determine the effect of treatment on the expression of inflammatory mRNAs, total RNA was extracted from oviducts ($n = 4$ mice per treatment) using TRIzol reagent (Invitrogen) and purified with RNeasy columns (Qiagen) according to the manufacturer's instructions and as described previously (Jeoung *et al.* 2010; Bridges *et al.* 2012). To determine the effect of treatment on the expression of miRNAs, total RNA was extracted from oviducts ($n = 4$ mice per treatment) using the miRNeasy Mini Kit (Qiagen) according to manufacturer's instructions. In each case, RNA quality was analysed by determining the RNA integrity number (RIN) using an Agilent 2100 Bioanalyzer (Agilent Technologies) at the University of Kentucky Microarray Core Facility. For all samples, RINs were greater than 9.7 (for mRNA analysis) and 9.8 (for miRNA analysis), and *28S/18S* rRNA absorbance ratios were greater than 2.0 (for mRNA analysis) and 1.7 (for miRNA analysis). All samples passed the quality control measurements outlined by Nanostring Technologies for their use within each platform.

Nanostring analysis of mRNA and miRNA

For the determination of inflammatory mRNAs, the nCounter GX Mouse Inflammation Kit (Nanostring Technologies) was used, which consists of 179 inflammation-related genes and six internal reference genes. For the determination of miRNAs, the nCounter Mouse v.1.5 miRNA Expression Assay (Nanostring Technologies) was used, which targets 578 mouse miRNAs, 33 viral miRNAs and four internal reference genes. For both mRNA and miRNA, analyses were performed according to the manufacturer's instructions at the University of Kentucky Microarray Core Facility. The digital multiplexed NanoString nCounter analysis system uses molecular barcodes to detect and count transcripts. Briefly, 100 ng total RNA from each sample was hybridised with reporter and capture probes that hybridise directly to target molecules. After hybridisation, sample processing allows for probe–target complexes to be immobilised on the nCounter cartridge and unbound probes removed. After sample processing, digital data acquisition allows for barcodes on reporter probes to be tabulated for each target molecule. The raw reported code count data generated from the nCounter Digital Analyzer was then exported to nSolver software (NanoString Technologies) for normalisation, background assessment and molecule count summarisation, as described previously (Koti *et al.* 2015). Following normalisation procedures, background adjustment and count summarisation, the resulting data were subjected to statistical analyses. Data were first tested for normality and homogeneity of variance using the Shapiro–Wilk (Shapiro and Wilk 1965) and Levene (Levene 1960) tests respectively. The data for 12 transcripts (chemokine (C-C motif) ligand 2 (*Ccl2*), chemokine (C-C motif) ligand 3 (*Ccl3*), chemokine (C-C motif) ligand 7 (*Ccl7*), chemokine (C-C motif) ligand 19 (*Ccl19*), chemokine (C-X-C motif) ligand 1 (*Cxcl1*), chemokine (C-X-C motif) ligand 5 (*Cxcl5*), chemokine (C-X-C motif) ligand 10 (*Cxcl10*), complement component 1, r subcomponent A (*C1r*), complement factor B (*Cfb*), colony stimulating factor 1 (*Csf1*), signal transducer and activator of transcription 1 (*Stat1*) and interleukin (IL)-23 receptor (*Il23r*)) were log transformed to attain normality and/or homogeneity of variance. Data that were normally distributed and with a homogeneous variance were then subjected to a one-way analysis of variance (ANOVA) to determine differences in relative molecule counts, with significance set to $P < 0.05$. If differences were detected, treatment means were separated using Scheffé's test to determine which means differed (Scheffé 1959). An

expression difference between treatments of $P < 0.05$ was considered statistically significant. With the goal of providing others full access to the data generated, complete data files have been deposited into the Gene Expression Omnibus (National Center for Biotechnology Information; Edgar *et al.* 2002) with the SuperSeries Accession no. GSE89096 (http://www.ncbi.nlm.nih.gov/geo, accessed 15 April 2017). Within this accession, data files for the mRNA and miRNA analyses are available as accessions GSE89095 and GSE89094 respectively.

Real-time reverse transcription–polymerase chain reaction analysis of mRNA

Real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed to confirm the level of expression of mRNAs encoding CCL5, CXCL1, CXCL10, Chemokine (C-X-C motif) receptor 2 (CXCR2, IL18RB) and IL-18 receptor accessory protein (IL18RAP), using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the housekeeping gene. Real-time RT-PCR was performed using an Eppendorf Mastercycler ep realplex^2 system with iQ SYBR Green Supermix (Bio-Rad), as described previously (Jeoung *et al.* 2010; Bridges *et al.* 2012). The following oligonucleotide primer pairs were used: for *Ccl5*, CCT CAC CAT ATG GCT CGG AC (forward) and ACG ACT GCA AGA TTG GAG CA (reverse); for *Cxcl1*, ACT CAA GAA TGG TCG CGA GG (forward) and GTG CCA TCA GAG CAG TCT GT (reverse); for *Cxcl10*, CTA TCC TGC CCA CGT GTT GA (forward) and TCC ACT GGG TAA AGG GGA GT (reverse); for *Cxcr2*, CTT AGC CAA GGA GGG AAG GC (forward) and GGG CTC TGC TAA GAA CGGT GA (reverse); for *Il18rap*, TGG AAT GAA GCG GCA TCT GT (forward) and CCG GTG ATT CTG TTC AGG CT (reverse); and for *Gapdh*, CCC CCA ATG TGT CCG TCG TGG (forward) and TGA GAG CAA TGC CAG CCC CG (reverse).

For each sample, cDNA was synthesised using the SuperScript III 1st Strand Synthesis System (Invitrogen), with 0.5 μg RNA used for each reverse transcription reaction. Real-time RT-PCR was performed in a total volume of 25 μL per reaction, with each reaction containing 5 μL cDNA, 1 μL of a 10 μM stock of each primer (forward and reverse), 12.5 μL of 2× SYBR Green PCR Master Mix and 5.5 μL nuclease-free water. The typical dissociation curves of these cDNA, plus *GAPDH* as the housekeeping gene, were confirmed and gene expression was analysed by the $2^{\Delta\Delta}$Cq method (Livak and Schmittgen 2001).

Results

NanoString nCounter gene expression analysis of inflammatory mRNA

Of the 179 targeted mRNAs within the nCounter GX Mouse Inflammation Kit, 49 were affected by treatment with LPS ($P < 0.05$). Among these 49 mRNAs, 14 transcripts encoded chemokine ligands and/or their receptors (Table 1). The level of expression of 13 of these 14 mRNAs was increased by treatment with 10 μg LPS. The level of expression of one mRNA (*Ccl22*) was increased by treatment with 2 μg LPS, but not by 10 μg LPS. Nine (of 49) transcripts affected by treatment with LPS encoded complement components (Table 1), with the level of expression of all nine of these mRNAs increased by treatment with 10 μg LPS. The remaining 26 of 49 transcripts affected by treatment with LPS encoded a variety of inflammatory proteins (Table 2). The level of expression of 24 of 26 of these mRNAs was increased by treatment with 10 μg LPS. The level of expression of one mRNA (phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide (*Pik3c2g*)) was increased by treatment with 2 but not 10 μg LPS, and the level of expression of one mRNA (src homology 2 domain-containing transforming protein C1, transcript variant 2 (*Shc1*)) was decreased by treatment with 2 μg LPS.

Real-time RT-PCR analysis of selected transcripts

Five mRNAs (*Ccl5*, *Cxcl1*, *Cxcl10*, *Cxcr2* and *Il18rap*) among the nanostring platform were selected for quantification by real-time RT-PCR. Comparisons of the effects of treatment on the expression of these mRNAs as determined by Nanostring analysis and real-time RT-PCR are available in Fig. S1, available as Supplementary Material. Although no formal statistical comparisons of the results from the two procedures were performed, the results obtained by NanoString and real-time RT-PCR appeared consistent.

NanoString nCounter analysis of miRNA

Of the 578 targeted mouse miRNAs within the nCounter Mouse v.1.5 miRNA Expression Assay, 51 were affected by LPS treatment (Table 3; $P < 0.05$). Treatment of mice with 2 μg LPS resulted in the differential expression of 36 miRNAs compared with treatment of mice with PBS alone. Of these 36 miRNAs, the expression of 20 was increased and the expression of 16 was decreased compared with PBS treatment alone. Treatment of mice with 10 μg LPS affected the expression of 45 of 51 differentially expressed miRNAs compared with PBS treatment alone. Of these 45 miRNAs, the expression of 27 was increased and the expression of 18 was decreased compared with PBS treatment alone.

Predicted miRNAs targeting mRNAs encoding chemokine and interleukin ligands

A prediction of the miRNAs targeting a subset of inflammatory mRNAs is included in this report. Using the microRNA.org database (Betel *et al.* 2010), targets of conserved miRNAs with an miRSVR score ≤0.01, a PhastCons score ≥0.0 and at least 6 mers seed complementarity are indicated. Of the 12 chemokine ligands with a C-C motif that were included in the nCounter GX Mouse Inflammation Kit, the level of expression of eight transcripts differed in the oviducts of mice treated with LPS ($P < 0.05$). For these 12 mRNAs, miRNA target prediction was performed, and the identities of the miRNAs that were affected by treatment with LPS and predicted as post-transcriptional modulators of each of these mRNAs are given in Table 4. Differentially expressed miRNAs were predicted to target 9 of 12 of these transcripts. In contrast, of the 18 mRNAs encoding ligands of the interleukin family that were included in the nCounter GX Mouse Inflammation Kit, the expression of only three transcripts differed in the oviducts of mice treated with LPS ($P < 0.05$). The identities of the miRNAs that were affected by treatment with LPS and predicted as post-transcriptional

Table 1. Expression of mRNAs encoding chemokines, complement components and/or their receptors that differed with lipopolysaccharide (LPS) treatment ($P < 0.05$)

Mice were treated with 0, 2 or 10 μg LPS in 100 μL phosphate-buffered saline on the morning of oestrus and killed 24 h later for extraction of RNA from oviducts and subsequent mRNA expression analysis. Data are provided as the least squares mean ± s.e.m. for each mRNA affected by LPS treatment. Model *P*-values are indicated from the one-way ANOVA used to determine an effect of treatment. If differences were detected, treatment means were separated using Scheffé's test ($P < 0.05$). Within rows, different superscripts indicate significant differences in the level of expression ($P < 0.05$)

Gene symbol	Gene description	*P*-value	LPS (μg)		
			0	2	10
Ccl2	Chemokine (C-C motif) ligand 2	<0.001	49.3 ± 7.2^{a}	119.3 ± 40.8^{a}	683.6 ± 92.2^{b}
Ccl3	Chemokine (C-C motif) ligand 3	0.006	8.8 ± 0.4^{a}	$17.6 \pm 3.3^{a,b}$	29.4 ± 6.5^{b}
Ccl4	Chemokine (C-C motif) ligand 4	0.003	8.8 ± 1.5^{a}	15.4 ± 3.0^{a}	30.5 ± 4.3^{b}
Ccl7	Chemokine (C-C motif) ligand 7	<0.001	8.6 ± 2.6^{a}	17.8 ± 6.9^{a}	152.6 ± 16.4^{b}
Ccl8	Chemokine (C-C motif) ligand 8	0.001	80.4 ± 28.9^{a}	382.3 ± 77.9^{a}	723.3 ± 110.5^{b}
Ccl17	Chemokine (C-C motif) ligand 17	0.038	17.1 ± 1.5^{a}	$19.1 \pm 3.4^{a,b}$	29.5 ± 3.7^{b}
Ccl19	Chemokine (C-C motif) ligand 19	0.006	58.0 ± 4.2^{a}	$84.0 \pm 16.9^{a,b}$	157.2 ± 25.3^{b}
Ccl22	Chemokine (C-C motif) ligand 22	0.017	10.9 ± 0.8^{a}	6.8 ± 0.6^{b}	$9.7 \pm 1.1^{a,b}$
Cxcl1	Chemokine (C-X-C motif) ligand 1	<0.001	9.9 ± 1.9^{a}	15.2 ± 3.5^{a}	70.3 ± 15.1^{b}
Cxcl2	Chemokine (C-X-C motif) ligand 2	0.049	1.5 ± 0.4^{a}	$4.1 \pm 1.4^{a,b}$	6.7 ± 1.6^{b}
Cxcl5	Chemokine (C-X-C motif) ligand 5	0.016	10.4 ± 5.2^{a}	$16.6 \pm 2.7^{a,b}$	68.3 ± 22.5^{b}
Cxcl10	Chemokine (C-X-C motif) ligand 10	<0.001	21.4 ± 4.1^{a}	72.1 ± 21.1^{b}	331.5 ± 42.6^{c}
Ccr7	Chemokine (C-C motif) receptor 7	0.03	2.8 ± 0.9^{a}	$9.8 \pm 3.1^{a,b}$	12.4 ± 1.9^{b}
Cxcr2	Chemokine (C-X-C motif) receptor 2	<0.001	4.5 ± 0.6^{a}	22.2 ± 3.9^{b}	48.4 ± 6.3c
C1s	Complement component 1, s subcomponent	<0.001	2745.7 ± 572.6^{a}	4998.2 ± 1065.8^{a}	12490.2 ± 1051.8^{b}
C1r	Complement component 1, r subcomponent A	<0.001	1064.3 ± 98.3^{a}	1815.7 ± 392.1^{a}	4605.7 ± 248.3^{b}
Cfb	Complement factor B	<0.001	81.8 ± 18.4^{a}	696.9 ± 316.9^{b}	2204.3 ± 289.8^{c}
C3	Complement component 3	<0.001	10832.8 ± 2251.4^{a}	18472.3 ± 3741.6^{a}	42404.1 ± 3318.1^{b}
C4a	Complement component 4A	0.001	871.8 ± 158.0^{a}	1353.5 ± 234.4^{a}	3073.6 ± 424.2^{b}
C2	Complement component 2	0.003	891.2 ± 134.8^{a}	1246.8 ± 249.4^{a}	2075.5 ± 118.4^{b}
C3ar1	Complement component 3a receptor 1	0.005	141.3 ± 22.0^{a}	$262.9 \pm 47.2^{a,b}$	340.9 ± 19.2^{b}
C1qb	Complement component 1, q subcomponent, β polypeptide	0.007	920.1 ± 77.2^{a}	$1802.0 \pm 427.1^{a,b}$	2487.4 ± 130.2^{b}
C1qa	Complement component 1, q subcomponent, α polypeptide	0.012	554.0 ± 56.4^{a}	$1227.1 \pm 324.4^{a,b}$	1614.9 ± 79.0^{b}

modulators of each of these 18 mRNAs are given in Table 5, with differentially expressed miRNAs predicted to target 13 of 18 of these transcripts.

Discussion

The aim of the present study was to determine acute LPS challenge-induced changes in the level of expression of inflammatory mRNAs and miRNAs in the mouse oviduct, increasing our understanding of the genetic regulation of inflammation in this reproductive organ. We hypothesised that systemic treatment with LPS would lead to an increase in the expression of mRNAs encoding inflammatory mediators in the oviduct. We found an effect of treatment on 49 of 179 targeted inflammatory mRNAs and on 51 of 578 miRNAs that may be acting as post-transcriptional regulators of inflammatory gene function. Overall, systemic treatment with LPS induced a rapid, robust inflammatory response in the mouse oviduct.

Treatment with LPS induced the differential expression of a range of mRNAs encoding chemokines, complement components, interleukins and tumour necrosis factor (TNF) family members, as well as several transcripts not easily classified within an inflammatory grouping. Chemokines are perhaps best recognised for their regulatory role in stimulating the migration of immune cells to, and within, an injured or inflamed tissue via chemotaxis (Fernandez and Lolis 2002; Le *et al.* 2004). Conversely, interleukins are widely considered as cytokines that modulate growth, differentiation and the activation of cells during the immune response (Brocker *et al.* 2010; Commin *et al.* 2010). The complement system acts as a bridge between innate and acquired immunity, functioning to recruit and enhance phagocytosis of target cells (Nesargikar *et al.* 2012), and the TNF superfamily can be broadly classified as a grouping of inflammatory cytokines that induce (in part through the activation of nuclear factor (NF)-κB) processes including cell proliferation, morphogenesis and apoptosis (Aggarwal *et al.* 2012). Overall, the response to an inflammatory insult is a multitiered and highly coordinated process.

Although LPS is widely used to induce an inflammatory response, to our knowledge very limited information regarding the effects of LPS on the mouse oviduct is available. Some consistency with the reported effect of treatment with LPS on bovine oviductal epithelial cells *in vitro* can be found, with LPS reported to increase the expression of *Il4*, *Il10*, *Il1β* and *TNFα* (Kowsar *et al.* 2013), as well as *Il1b*, *TNFα* and *TGFβ1* (Ibrahim *et al.* 2015) mRNAs in bovine oviductal epithelial cells. We observed increased expression of *Il1b*, *Il7*, *Il15*, *TGFβ1* and *TNF* mRNAs after systemic treatment of mice with LPS *in vivo*.

Among the chemokines, our nanostring analysis detected an increase in the level of expression of *Ccl2*, *Ccl3*, *Ccl4*, *Ccl7*,

Table 2. Expression of mRNAs encoding other inflammatory factors and proteins that differed with lipopolysaccharide (LPS) treatment ($P < 0.05$)
Mice were treated with 0, 2 or 10 µg LPS in 100 µL phosphate-buffered saline on the morning of oestrus and killed 24 h later for extraction of RNA from oviducts and subsequent mRNA expression analysis. Data are provided as the least squares mean ± s.e.m. for each mRNA affected by LPS treatment. Model *P*-values are indicated from the one-way ANOVA used to determine an effect of treatment. If differences were detected, treatment means were separated using Scheffé's test ($P < 0.05$). Within rows, different superscripts indicate significant differences in the level of expression ($P < 0.05$). TNFRSF, tumor necrosis factor receptor superfamily

Gene symbol	Gene description	*P*-value	LPS (µg)		
			0	2	10
Csf1	Colony stimulating factor 1	<0.001	365.0 ± 18.8[a]	367.5 ± 32.1[a]	703.2 ± 65.0[b]
Stat1	Signal transducer and activator of transcription 1	<0.001	687.0 ± 76.6[a]	951.2 ± 141.4[a]	2302.4 ± 374.5[b]
Cd40	Tumour necrosis factor receptor superfamily member 5	<0.001	21.5 ± 0.6[a]	30.3 ± 6.1[a]	60.6 ± 2.6[b]
Il15	Interleukin 15	0.001	34.4 ± 1.4[a]	42.3 ± 2.3[a]	62.0 ± 5.1[b]
Tnfsf14	Tumour necrosis factor (ligand) superfamily, member 14	0.002	6.5 ± 0.9[a]	10.0 ± 1.1[a]	15.7 ± 1.7[b]
Il18rap	Interleukin 18 receptor accessory protein	0.003	6.4 ± 1.0[a]	12.3 ± 0.9[a,b]	19.5 ± 3.0[b]
Tgfb1	Transforming growth factor, β1	0.005	262.7 ± 11.2[a]	275.1 ± 26.4[a]	377.2 ± 18.8[b]
Ly96	Lymphocyte Antigen 96	0.006	214.8 ± 10.0[a]	242.6 ± 19.3[a,b]	295.5 ± 8.4[b]
Il1b	Interleukin 1β	0.007	22.7 ± 5.2[a]	31.8 ± 5.4[a]	55.7 ± 6.1[b]
Tnf	Tumour necrosis factor	0.012	6.3 ± 1.9[a]	10.0 ± 4.8[a]	26.3 ± 4.3[b]
Cebpb	CCAAT/enhancer binding protein (C/EBP), β	0.014	1296.0 ± 115.5[a]	1299.4 ± 71.3[a]	1715.9 ± 76.8[b]
Mafk	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein K	0.014	142.0 ± 13.2[a]	148.5 ± 13.4[a]	200.2 ± 8.6[b]
Ltb	Lymphotoxin B	0.014	64.6 ± 5.0[a]	88.9 ± 14.5[a,b]	134.3 ± 17.0[b]
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.020	671.3 ± 52.0[a]	732.5 ± 28.2[a,b]	842.3 ± 11.1[b]
Mapkapk2	Mitogen-activated protein kinase-activated protein kinase 2	0.021	1432.1 ± 25.4[a]	1565.5 ± 64.1[a,b]	1723.4 ± 75.0[b]
Itgb2	Integrin β 2	0.021	100.3 ± 10.4[a]	182.7 ± 37.4[a,b]	242.6 ± 31.4[b]
Daxx	Fas death domain-associated protein	0.026	78.8 ± 4.7[a,b]	73.6 ± 3.8[a]	90.3 ± 1.7[b]
Nos2	Nitric oxide synthase 2, inducible	0.027	8.2 ± 1.5[a]	10.3 ± 2.1[a,b]	15.8 ± 1.4[b]
Maff	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F	0.027	113.3 ± 26.4[a]	136.0 ± 8.9[a,b]	189.8 ± 7.8[b]
Il7	Interleukin 7	0.028	36.4 ± 2.1[a,b]	34.4 ± 3.1[a]	46.8 ± 3.2[b]
Shc1	src homology 2 domain-containing transforming protein C1, transcript variant 2	0.028	1711.4 ± 34.7[a]	1537.7 ± 52.6[b]	1651.3 ± 18.2[a,b]
Pik3c2g	Phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide	0.037	7.6 ± 0.6[a,b]	11.4 ± 2.0[a]	4.9 ± 1.5[b]
Gusb	Glucuronidase, β	0.038	414.0 ± 12.8[a]	437.0 ± 21.3[a,b]	490.7 ± 18.8[b]
Ripk2	Receptor (TNFRSF)-interacting serine-threonine kinase 2	0.045	169.2 ± 15.9[a]	224.1 ± 15.5[a,b]	254.6 ± 27.6[b]
Il23r	Interleukin 23 receptor	0.046	8.4 ± 1.4[a]	10.0 ± 0.9[a]	23.9 ± 3.5[b]
Prkcb1	Protein kinase C, β1	0.047	125.1 ± 7.1[a]	127.3 ± 11.3[a,b]	162.2 ± 10.9[b]

Ccl8, *Ccl17*, *Ccl19*, *Ccl22*, *Cxcl1*, *Cxcl2*, *Cxcl5*, *Cxcl10*, *Ccr7* and *Cxcr2* (*Il18rb*) mRNAs in the oviducts of mice treated with 10 µg LPS. In addition, targeted RT-PCR revealed an LPS-induced increase in the level of expression of *Ccl5* mRNA. The expression of mRNA encoding CCL5 and CXCL9 has been reported to increase in the oviducts of *C. trachomatis*-infected mice (Maxion and Kelly 2002), and serum concentrations of CCL5 (also known as regulated upon activation, normal T expressed and secreted (RANTES)) are reported to increase in women with antibodies indicative of previous infection with *C. trachomatis* (Shibahara *et al.* 2003), suggesting insult-specific induction of this class of inflammatory mediators. The relative level of *Cxcl1* mRNA in the upper genital tract of mice exposed to *Chlamydia muridarum* is reported to differ with the variant of *C. muridarum* used (Yeruva *et al.* 2014), and CXCL1-deficient mice exhibit a decreased white blood cell and neutrophil count in response to challenge with *Klebsiella pneumoniae* (Batra *et al.* 2012). Overall, CXCL1 is associated with Gram-negative bacterial infections, exerting neutrophil attractant activity through the chemokine receptor CXCR2, also referred to as IL18RB (Jin *et al.* 2014). Increases in the expression of CXCR2 have also been suggested to be associated with inflammation-induced tubal ectopic pregnancies in women, but it has not been determined whether CXCR2 expression plays a role in the development of ectopic pregnancy or is induced by ectopic pregnancy (Balasubramaniam *et al.* 2012).

Increasing attention is now being given to post-transcriptional modification by miRNAs. However, a great deal more research is required before the full effects of these small non-coding RNAs on gene and protein expression are delineated. For example, we detected mmu-let-7c (with the mature sequence now identified as mmu-let-7c-5p) as a highly abundant LPS-induced miRNA. The microRNA.org database (Betel *et al.* 2010) predicted >5000 targeted mRNAs for this miRNA, and TarBase v7.0 from the DIANA laboratory group (Vlachos *et al.* 2015), when set with a high precision miRNA targeted gene (miTG) score of >0.6, revealed >400 validated mRNA targets. With our interest in inflammation, we therefore used an alternative approach and predicted miRNA targets for a selection of the inflammatory mRNAs that were included in the nanostring panel. Among these, *Ccl22*, *Il1a*, *Il6*, *Il10* and *Il13* mRNAs were all predicted as targets of the LPS-induced mmu-let-7c.

In LPS-treated bovine oviductal epithelial cells, a decrease in the abundance of miR-146a, miR-155, miR-21 and miR-223 has

Table 3. Expression of microRNAs (miRNAs) that differed with lipopolysaccharide (LPS) treatment ($P < 0.05$)

Mice were treated with 0, 2 or 10 µg LPS in 100 µL phosphate-buffered saline on the morning of oestrus and killed 24 h later for extraction of RNA from oviducts and subsequent miRNA (miR) expression. Data are provided as the least squares mean ± s.e.m. for each miRNA affected by LPS treatment. Model P-values are indicated from the one-way ANOVA used to determine an effect of treatment. If differences were detected, treatment means were separated using Scheffé's test ($P < 0.05$). Within rows, different superscripts indicate significant differences in the level of expression ($P < 0.05$)

miR identity	P-value	LPS (µg)		
		0	2	10
mmu-miR-203	<0.001	116.8 ± 6.2[a]	207.0 ± 6.6[b]	365.0 ± 23.6[c]
mmu-miR-23a	<0.001	7295.9 ± 251.7[a]	5518.8 ± 137.0[b]	4719.8 ± 127.4[c]
mmu-miR-101a	<0.001	97.6 ± 8.3[a]	186.3 ± 20.2[b]	294.3 ± 17.9[c]
mmu-miR-1942	<0.001	51.2 ± 1.4[a]	65.3 ± 2.0[b]	71.9 ± 2.1[c]
mmu-miR-322	<0.001	2383.3 ± 164.0[a]	1588.8 ± 60.9[b]	1231.3 ± 124.1[b]
mmu-miR-30c	0.001	6689.7 ± 192.4[a]	4730.2 ± 209.1[b]	4507.1 ± 227.6[b]
mmu-miR-30b	0.001	2138.3 ± 161.2[a]	2836.3 ± 108.5[b]	3414.1 ± 152.7[c]
mmu-miR-1896	0.001	354.3 ± 5.1[a]	317.8 ± 5.1[b]	306.0 ± 5.9[b]
mmu-miR-1187	0.001	45.8 ± 1.7[a]	38.9 ± 1.1[a]	58.5 ± 3.4[b]
mmu-miR-222	0.001	129.7 ± 6.9[a]	247.3 ± 11.4[b]	258.6 ± 22.4[b]
mmu-miR-101b	0.002	156.9 ± 6.9[a]	211.1 ± 16.6[a]	313.6 ± 25.2[b]
mmu-miR-26b	0.002	1796.6 ± 77.7[a]	2132.5 ± 78.7[b]	2520.6 ± 101.9[c]
mmu-miR-27a	0.003	5932.2 ± 129.0[a]	4225.2 ± 124.4[b]	4524.8 ± 328.6[b]
mmu-miR-30e	0.003	396.8 ± 17.9[a]	519.5 ± 23.8[b]	558.6 ± 20.7[b]
mmu-miR-375	0.004	1377.9 ± 19.7[a]	901.5 ± 133.7[b]	557.3 ± 116.1[b]
mmu-miR-26a	0.004	224.8 ± 14.0[a]	349.5 ± 29.6[b]	452.5 ± 38.2[c]
mmu-miR-29a	0.004	26 879 ± 1210[a]	30 131 ± 657[b]	33 013 ± 795[c]
mmu-miR-708	0.004	91.6 ± 3.4[a]	112.5 ± 1.4[b]	98.5 ± 3.9[a]
mmu-miR-1941-3p	0.006	57.4 ± 2.9[a]	54.0 ± 2.5[a]	67.9 ± 1.7[b]
mmu-miR-1927	0.006	51.5 ± 3.4[a]	40.2 ± 2.2[b]	34.6 ± 2.2[b]
mmu-miR-196b	0.006	818.4 ± 130.9[a]	1305.8 ± 53.4[b]	1402.8 ± 98.9[b]
mmu-miR-350	0.007	381.5 ± 6.4[a]	324.7 ± 4.9[b]	351.8 ± 11.9[b]
mmu-let-7c	0.007	20 207 ± 1109[a]	25 189 ± 1251[a]	33 816 ± 3079[b]
mmu-miR-151-5p	0.009	1353.8 ± 44.2[a]	1151.3 ± 41.5[b]	1082.5 ± 46.5[b]
mmu-miR-1186	0.01	586.5 ± 9.5[a]	524.5 ± 11.2[b]	505.0 ± 17.2[b]
mmu-miR-146a	0.011	883.2 ± 44.4[a]	1589.5 ± 114.6[a]	2480.6 ± 404.5[b]
mmu-miR-421	0.011	57.1 ± 3.2[a]	84.9 ± 7.6[b]	86.4 ± 2.9[b]
mmu-miR-149	0.011	90.7 ± 12.0[a]	48.4 ± 6.4[b]	44.6 ± 7.9[b]
mmu-miR-155	0.012	1.0 ± 0.0[a]	56.5 ± 22.6[a]	150.4 ± 34.0[b]
mmu-miR-676	0.013	228.1 ± 10.7[a]	184.1 ± 8.8[b]	172.4 ± 10.5[b]
mmu-miR-19a	0.013	545.9 ± 19.3[a]	676.0 ± 30.1[b]	695.9 ± 27.9[b]
mmu-miR-151-3p	0.015	90.4 ± 4.9[a]	70.6 ± 3.7[b]	71.3 ± 3.4[b]
mmu-miR-99b	0.023	680.1 ± 40.5[a]	820.3 ± 35.0[a,b]	925.1 ± 58.3[b]
mmu-miR-429	0.023	4398.2 ± 305.4[a]	3444.9 ± 199.6[b]	4295.2 ± 153.8[a]
mmu-miR-691	0.025	22.7 ± 1.2[a]	27.4 ± 5.3[a]	41.7 ± 3.3[b]
mmu-miR-762	0.027	43.6 ± 1.1[a]	59.0 ± 5.3[b]	61.3 ± 2.4[b]
mmu-miR-1900	0.031	272.4 ± 6.0[a]	264.1 ± 4.4[a]	243.7 ± 7.6[b]
mmu-miR-32	0.033	116.7 ± 8.0[a]	147.2 ± 1.8[b]	149.9 ± 10.1[b]
mmu-miR-16	0.034	15 154 ± 812[a]	14 239 ± 282[a]	12 103 ± 835[b]
mmu-miR-374	0.034	271.2 ± 20.9[a]	370.2 ± 26.7[b]	354.7 ± 15.5[b]
mmu-let-7f	0.035	5838.3 ± 238.2[a]	7262.8 ± 379.1[b]	7778.9 ± 501.8[b]
mmu-miR-106a + mmu-miR-17	0.035	1096.4 ± 36.4[a]	993.5 ± 19.4[a,b]	928.7 ± 44.9[b]
mmu-miR-139-5p	0.036	99.4 ± 4.5[a]	113.3 ± 7.3[a,b]	134.3 ± 8.8[b]
mmu-miR-218	0.038	768.7 ± 17.0[a]	719.7 ± 8.6[b]	757.8 ± 10.0[a]
mmu-miR-467e	0.039	55.3 ± 3.0[a]	64.5 ± 5.2[a,b]	74.6 ± 3.4[b]
mmu-miR-434-5p	0.04	39.0 ± 4.4[a]	55.7 ± 3.3[b]	48.1 ± 3.4[a,b]
mmu-miR-130a	0.042	3377.4 ± 102.1[a]	3260.1 ± 90.5[a,b]	2986.8 ± 80.7[b]
mmu-miR-664	0.042	41.7 ± 2.2[a]	44.6 ± 1.9[a,b]	50.4 ± 1.9[b]
mmu-miR-301b	0.042	100.9 ± 7.4[a]	131.7 ± 2.1[b]	121.0 ± 9.1[a,b]
mmu-miR-335-5p	0.042	169.1 ± 18.8[a]	127.2 ± 9.4[b]	116.9 ± 9.2[b]
mmu-miR-129-3p	0.046	277.1 ± 34.0[a]	396.8 ± 25.4[b]	364.5 ± 24.7[a,b]

Table 4. Predicted regulation of C-C motif chemokine ligands by microRNAs (miRNAs) that differed with lipopolysaccharide treatment ($P < 0.05$)

Target prediction was performed using the microRNA.org database. For each mRNA, only predicted miRNAs that differed with treatment are indicated. Only conserved miRNAs with an miRSVR score ≤0.01, a PhastCons score ≥0.0 and at least 6 mers seed complementarity are included. For each mRNA of $P < 0.05$, P-values represent the significance of treatment means that were separated using Scheffé's test. NS, not significant ($P \geq 0.05$). Gene symbols are defined in Table 1

Gene symbol	*P*-value	Identity of differentially expressed miRNAs
Ccl2	<0.001	miR-27a
Ccl3	0.006	miR26a, miR-26b
Ccl4	0.003	miR-27a, miR-429
Ccl5	NS	–
Ccl7	<0.001	miR-101a, miR-101b, miR-19a, miR-23a, miR-421
Ccl8	0.001	miR-146a, miR-375
Ccl11	NS	miR-19a, miR-335-5p, miR-374
Ccl17	0.038	miR-301b
Ccl19	0.006	miR-130a, miR-301b
Ccl21b	NS	–
Ccl22	0.017	let-7c, let-7f, miR-27a, miR-374
Ccl24	NS	–

Table 5. Predicted regulation of interleukin (Il) ligands by microRNAs (miRNAs) that differed with lipopolysaccharide treatment ($P < 0.05$)

Target prediction was performed using the microRNA.org database. For each mRNA, only predicted miRNAs that differed with treatment are indicated. Only conserved miRNAs with an miRSVR score ≤0.01, a PhastCons score ≥0.0 and at least 6 mers seed complementarity are included. For each mRNA of $P < 0.05$, P-values represent the significance of treatment means that were separated using Scheffé's test. NS, not significant ($P \geq 0.05$)

Gene symbol	*P*-value	Identity of differentially expressed miRNAs
Il1a	NS	let-7c, let-7f, miR-27a, miR-30b, miR-30c, miR-30e, miR-218, miR-421
Il1b	0.007	miR-203
Il2	NS	miR-30b, miR-30c, miR-30e, miR-374, miR-429
Il3	NS	–
Il4	NS	–
Il5	NS	miR-149, miR-222
Il6	NS	let-7c, let-7f, miR-26a, miR-26b, miR-30b, miR-30c, miR-30e, miR-149, miR-374
Il7	0.028	miR-130a, miR-146a, miR-355-5p, miR-429
Il9	NS	–
Il10	NS	let-7c, let-7f, miR-32, miR-374, miR-429, miR-708
Il11	NS	–
Il12a	NS	let-7c, let-7f, miR-374, miR-708
Il12b	NS	miR-149, miR-203, miR-421
Il13	NS	let-7c, let-7f, miR-139-5p, miR-429
Il15	0.001	miR-16, miR-322
Il18	NS	–
Il22	NS	miR-203, miR-374, miR-375
Il23a	NS	miR-27a

been reported (Ibrahim *et al.* 2015). In the present study, we observed an LPS-induced increase in the abundance of miR-146a and miR-155 in the oviducts of mice, with no effect of treatment on miR-21 or miR-223. Predicted targets for miR-146a were *Ccl8* and *Il7* mRNAs, and CCL8/miR-146a has been proposed as a prognostic marker for human melanoma (Barbai *et al.* 2015). Although none of the mRNAs we analysed were predicted as targets for miR-155, transcriptome profiling of bone marrow-derived macrophages from wild-type and miR-155-knockout mice has revealed this miRNA as a major regulator of the inflammatory M1 macrophage response (Jablonski *et al.* 2016). A defined role for miR-21 in coordinating the early innate immune response to LPS-induced peritonitis has also been reported (Barnett *et al.* 2016). The relationship and role of these miRNAs in regulating the oviducts response to an inflammatory insult are still to be determined.

The differential expression of miRNAs within the oviduct of women with ectopic pregnancies has also been reported (Feng *et al.* 2014). Feng *et al.* (2014) reported that four miRNAs could identify tubal implantation from non-implantation sites in women. Of these four, we observed an LPS-induced decrease in the abundance of one miRNA (miR-149), with identified predictive targets including *Il5* and *Il6* mRNAs. Immunohistochemistry has revealed increased expression of IL-6 near the implantation site in women with tubal pregnancies (Balasubramaniam *et al.* 2012). Taking IL6 further, we did not observe an effect of treatment on the expression of *Il6* mRNA, but we did find an effect of treatment on 12 miRNAs predicted as post-transcriptional regulators of this inflammatory interleukin (Table 5). Further indicative of the rapidly expanding field of miRNA research, in one recent report that used miR-21-knockout mice, genetic deletion of miR-21 resulted in increased LPS-stimulated bone marrow-derived macrophage levels of IL6 (Barnett *et al.* 2016). Our target prediction for *Il6* did not identify miR-21 among the predicted targets; it appears likely that target prediction databases will in the future.

Understanding the genetic regulation of the process of inflammation in the oviduct is instrumental to improving the reproductive health of woman. The present study provides a comprehensive profile of acute LPS-induced inflammatory mRNAs and miRNAs in the oviducts of mice and our current knowledge of predicted targets for a subset of the mRNAs we evaluated.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This study was supported by National Institutes of Health Grant P01 HD071875 (to Phillip J. Bridges), the University of Kentucky and Kentucky Agricultural Experiment Station. The information reported in this paper (Publication no. 17-07-091) is part of a project of the Kentucky Agricultural Experiment Station and is published with the approval of the Director.

References

Aggarwal, B. B., Gupta, S. C., and Kim, J. H. (2012). Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. *Blood* **119**, 651–665. doi:10.1182/BLOOD-2011-04-325225

Balasubramaniam, E. S., Van Noorden, S., and El-Bahrawy, M. (2012). The expression of interleukin (IL)-6, IL-8, and their receptors in fallopian tubes with ectopic tubal gestation. *Fertil. Steril.* **98**, 898–904. doi:10.1016/J.FERTNSTERT.2012.06.004

Barbai, T., Fejos, Z., Puskas, L. G., Timar, J., and Raso, E. (2015). The importance of microenvironment: the role of CCL8 in metastasis formation of melanoma. *Oncotarget* **6**, 29111–29128. doi:10.18632/ONCOTARGET.5059

Barnett, R. E., Conklin, D. J., Ryan, L., Keskey, R. C., Ramjee, V., Sepulveda, E. A., Srivastava, S., Bhatnagar, A., and Cheadle, W. G. (2016). Anti-inflammatory effects of miR-21 in the macrophage response to peritonitis. *J. Leukoc. Biol.* **99**, 361–371. doi:10.1189/JLB.4A1014-489R

Batra, S., Cai, S., Balamayooran, G., and Jeyaseelan, S. (2012). Intrapulmonary administration of leukotriene B(4) augments neutrophil accumulation and responses in the lung to *Klebsiella* infection in CXCL1 knockout mice. *J. Immunol.* **188**, 3458–3468. doi:10.4049/JIMMUNOL.1101985

Betel, D., Koppal, A., Agius, P., Sander, C., and Leslie, C. (2010). Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol.* **11**, R90. doi:10.1186/GB-2010-11-8-R90

Bridges, P. J., Jeoung, M., Shim, S., Park, J. Y., Lee, J. E., Sapsford, L. A., Trudgen, K., Ko, C., Gye, M. C., and Jo, M. (2012). Hematopoetic prostaglandin D synthase: an ESR1-dependent oviductal epithelial cell synthase. *Endocrinology* **153**, 1925–1935. doi:10.1210/EN.2011-1900

Brocker, C., Thompson, D., Matsumoto, A., Nebert, D. W., and Vasiliou, V. (2010). Evolutionary divergence and functions of the human interleukin (IL) gene family. *Hum. Genomics* **5**, 30–55. doi:10.1186/1479-7364-5-1-30

Caligioni, C. S. (2009). Assessing reproductive status/stages in mice. *Curr. Protoc. Neurosci.* **Appendix 4**, Appendix 4I. doi:10.1002/0471142301.NSA04IS48

Centers for Disease Control and Prevention (2016). 'Sexually Transmitted Diseases Surveillance 2015.' (U.S. Department of Health and Human Services: Atlanta.)

Commins, S. P., Borish, L., and Steinke, J. W. (2010). Immunologic messenger molecules: cytokines, interferons, and chemokines. *J. Allergy Clin. Immunol.* **125**(Suppl. 2), S53–S72. doi:10.1016/J.JACI.2009.07.008

Creanga, A. A., Shapiro-Mendoza, C. K., Bish, C. L., Zane, S., Berg, C. J., and Callaghan, W. M. (2011). Trends in ectopic pregnancy mortality in the United States: 1980–2007. *Obstet. Gynecol.* **117**, 837–843. doi:10.1097/AOG.0B013E3182113C10

Darville, T., Andrews, C. W., Jr, Laffoon, K. K., Shymasani, W., Kishen, L. R., and Rank, R. G. (1997). Mouse strain-dependent variation in the course and outcome of chlamydial genital tract infection is associated with differences in host response. *Infect. Immun.* **65**, 3065–3073.

Darville, T., Andrews, C. W., Jr, Sikes, J. D., Fraley, P. L., Braswell, L., and Rank, R. G. (2001). Mouse strain-dependent chemokine regulation of the genital tract T helper cell type 1 immune response. *Infect. Immun.* **69**, 7419–7424. doi:10.1128/IAI.69.12.7419-7424.2001

Deb, K., Chaturvedi, M. M., and Jaiswal, Y. K. (2004). A 'minimum dose' of lipopolysaccharide required for implantation failure: assessment of its effect on the maternal reproductive organs and interleukin-1alpha expression in the mouse. *Reproduction* **128**, 87–97. doi:10.1530/REP.1.00110

Deguchi, T., Nakane, K., Yasuda, M., and Maeda, S. (2010). Emergence and spread of drug resistant *Neisseria gonorrhoeae*. *J. Urol.* **184**, 851–858. doi:10.1016/J.JURO.2010.04.078

Diamond, A. K., Sweet, L. M., Oppenheimer, K. H., Bradley, D. F., and Phillippe, M. (2007). Modulation of monocyte chemotactic protein-1 expression during lipopolysaccharide-induced preterm delivery in the pregnant mouse. *Reprod. Sci.* **14**, 548–559. doi:10.1177/1933719107307792

Donnez, J., and Casanas-Roux, F. (1988). Histology: a prognostic factor in proximal tubal occlusion. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **29**, 33–38. doi:10.1016/0028-2243(88)90162-1

Edgar, R., Domrachev, M., and Lash, A. E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* **30**, 207–210. doi:10.1093/NAR/30.1.207

Feinen, B., and Russell, M. W. (2012). Contrasting roles of IL-22 and IL-17 in murine genital tract infection by *Neisseria gonorrhoeae*. *Front. Immunol.* **3**, 11. doi:10.3389/FIMMU.2012.00011

Feng, Y., Zou, S., Weijdegard, B., Chen, J., Cong, Q., Fernandez-Rodriguez, J., Wang, L., Billig, H., and Shao, R. (2014). The onset of human ectopic pregnancy demonstrates a differential expression of miRNAs and their cognate targets in the Fallopian tube. *Int. J. Clin. Exp. Pathol.* **7**, 64–79.

Fernandez, E. J., and Lolis, E. (2002). Structure, function, and inhibition of chemokines. *Annu. Rev. Pharmacol. Toxicol.* **42**, 469–499. doi:10.1146/ANNUREV.PHARMTOX.42.091901.115838

García-Ulloa, A. C., and Arrieta, O. (2005). Tubal occlusion causing infertility due to an excessive inflammatory response in patients with predisposition for keloid formation. *Med. Hypotheses* **65**, 908–914. doi:10.1016/J.MEHY.2005.03.031

Hobbs, M. M., Anderson, J. E., Balthazar, J. T., Kandler, J. L., Carlson, R. W., Ganguly, J., Begum, A. A., Duncan, J. A., Lin, J. T., Sparling, P. F., Jerse, A. E., and Shafer, W. M. (2013). Lipid A's structure mediates *Neisseria gonorrhoeae* fitness during experimental infection of mice and men. *MBio* **4**, e00892-13. doi:10.1128/MBIO.00892-13

Ibrahim, S., Salilew-Wondim, D., Rings, F., Hoelker, M., Neuhoff, C., Tholen, E., Looft, C., Schellander, K., and Tesfaye, D. (2015). Expression pattern of inflammatory response genes and their regulatory microRNAs in bovine oviductal cells in response to lipopolysaccharide: implication for early embryonic development. *PLoS One* **10**, e0119388. doi:10.1371/JOURNAL.PONE.0119388

Igietseme, J. U., Omosun, Y., Partin, J., Goldstein, J., He, Q., Joseph, K., Ellerson, D., Ansari, U., Eko, F. O., Bandea, C., Zhong, G., and Black, C. M. (2013). Prevention of *Chlamydia*-induced infertility by inhibition of local caspase activity. *J. Infect. Dis.* **207**, 1095–1104. doi:10.1093/INFDIS/JIT009

Jablonski, K. A., Gaudet, A. D., Amici, S. A., Popovich, P. G., and Guerau-de-Arellano, M. (2016). Control of the inflammatory macrophage transcriptional signature by miR-155. *PLoS One* **11**, e0159724. doi:10.1371/JOURNAL.PONE.0159724

Jeoung, M., and Bridges, P. J. (2011). Cyclic regulation of apoptotic gene expression in the mouse oviduct. *Reprod. Fertil. Dev.* **23**, 638–644. doi:10.1071/RD11011

Jeoung, M., Lee, S., Hawng, H. K., Cheon, Y. P., Jeong, Y. K., Gye, M. C., Iglarz, M., Ko, C., and Bridges, P. J. (2010). Identification of a novel role for endothelins within the oviduct. *Endocrinology* **151**, 2858–2867. doi:10.1210/EN.2009-1155

Jin, L., Batra, S., Douda, D. N., Palaniyar, N., and Jeyaseelan, S. (2014). CXCL1 contributes to host defense in polymicrobial sepsis via modulating T cell and neutrophil functions. *J. Immunol.* **193**, 3549–3558. doi:10.4049/JIMMUNOL.1401138

Kessler, M., Zielecki, J., Thieck, O., Mollenkopf, H. J., Fotopoulou, C., and Meyer, T. F. (2012). *Chlamydia trachomatis* disturbs epithelial tissue homeostasis in fallopian tubes via paracrine Wnt signaling. *Am. J. Pathol.* **180**, 186–198. doi:10.1016/J.AJPATH.2011.09.015

Koomey, M. (2001). Implications of molecular contacts and signaling initiated by *Neisseria gonorrhoeae*. *Curr. Opin. Microbiol.* **4**, 53–57. doi:10.1016/S1369-5274(00)00164-8

Koti, M., Siu, A., Clement, I., Bidarimath, M., Turashvili, G., Edwards, A., Rahimi, K., Masson, A. M., and Squire, J. A. (2015). A distinct pre-existing inflammatory tumour microenvironment is associated with chemotherapy resistance in high-grade serous epithelial ovarian cancer. *Br. J. Cancer* **112**, 1215–1222. doi:10.1038/BJC.2015.81

Kowsar, R., Hambruch, N., Liu, J., Shimizu, T., Pfarrer, C., and Miyamoto, A. (2013). Regulation of innate immune function in bovine oviduct epithelial cells in culture: the homeostatic role of epithelial cells in balancing Th1/Th2 response. *J. Reprod. Dev.* **59**, 470–478. doi:10.1262/JRD.2013-036

Le, Y., Zhou, Y., Iribarren, P., and Wang, J. (2004). Chemokines and chemokine receptors: their manifold roles in homeostasis and disease. *Cell. Mol. Immunol.* **1**, 95–104.

Levene, H. (1960). 'Contributions to Probability and Statistics: Essays in Honor of Harold Hotelling.' (Eds I. Olkin *et al.*) pp. 278–292. (Stanford University Press: Stanford, CA.)

Liu, Y., Egilmez, N. K., and Russell, M. W. (2013). Enhancement of adaptive immunity to *Neisseria gonorrhoeae* by local intravaginal administration of microencapsulated interleukin 12. *J. Infect. Dis.* **208**, 1821–1829. doi:10.1093/INFDIS/JIT354

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402–408. doi:10.1006/METH.2001.1262

Mårdh, P. A. (2004). Tubal factor infertility, with special regard to chlamydial salpingitis. *Curr. Opin. Infect. Dis.* **17**, 49–52. doi:10.1097/00001432-200402000-00010

Maxion, H. K., and Kelly, K. A. (2002). Chemokine expression patterns differ within anatomically distinct regions of the genital tract during *Chlamydia trachomatis* infection. *Infect. Immun.* **70**, 1538–1546. doi:10.1128/IAI.70.3.1538-1546.2002

Mayorga, M., Iborra, A., Estany, S., and Martinez, P. (2004). Protective effect of vitamin E in an animal model of LPS-induced inflammation. *Am. J. Reprod. Immunol.* **52**, 356–361. doi:10.1111/J.1600-0897.2004.00233.X

Nesargikar, P. N., Spiller, B., and Chavez, R. (2012). The complement system: history, pathways, cascade and inhibitors. *Eur. J. Microbiol. Immunol. (Bp.)* **2**, 103–111. doi:10.1556/EUJMI.2.2012.2.2

Perfettini, J. L., Darville, T., Gachelin, G., Souque, P., Huerre, M., Dautry-Varsat, A., and Ojcius, D. M. (2000). Effect of *Chlamydia trachomatis* infection and subsequent tumor necrosis factor alpha secretion on apoptosis in the murine genital tract. *Infect. Immun.* **68**, 2237–2244. doi:10.1128/IAI.68.4.2237-2244.2000

Phillippe, M., Diamond, A. K., Sweet, L. M., Oppenheimer, K. H., and Bradley, D. F. (2011). Expression of coagulation-related protein genes during LPS-induced preterm delivery in the pregnant mouse. *Reprod. Sci.* **18**, 1071–1079. doi:10.1177/1933719111404607

Punnonen, R., Soderstrom, K. O., and Alanen, A. (1984). Isthmic tubal occlusion: etiology and histology. *Acta Eur. Fertil.* **15**, 39–42.

Raetz, C. R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71**, 635–700. doi:10.1146/ANNUREV.BIOCHEM.71.110601.135414

Rasmussen, S. J., Eckmann, L., Quayle, A. J., Shen, L., Zhang, Y. X., Anderson, D. J., Fierer, J., Stephens, R. S., and Kagnoff, M. F. (1997). Secretion of proinflammatory cytokines by epithelial cells in response to *Chlamydia* infection suggests a central role for epithelial cells in chlamydial pathogenesis. *J. Clin. Invest.* **99**, 77–87. doi:10.1172/JCI119136

Scheffé, H. (1959). 'The Analysis of Variance.' (Wiley: New York.)

Shao, R. (2010). Understanding the mechanisms of human tubal ectopic pregnancies: new evidence from knockout mouse models. *Hum. Reprod.* **25**, 584–587. doi:10.1093/HUMREP/DEP438

Shapiro, S. S., and Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika* **52**, 591–611. doi:10.1093/BIOMET/52.3-4.591

Shaw, J. L., Dey, S. K., Critchley, H. O., and Horne, A. W. (2010). Current knowledge of the aetiology of human tubal ectopic pregnancy. *Hum. Reprod. Update* **16**, 432–444. doi:10.1093/HUMUPD/DMP057

Shibahara, H., Hirano, Y., Ayustawati, , Kikuchi, K., Taneichi, A., Fujiwara, H., Takamizawa, S., and Sato, I. (2003). Chemokine bioactivity of RANTES is elevated in the sera of infertile women with past *Chlamydia trachomatis* infection. *Am. J. Reprod. Immunol.* **49**, 169–173. doi:10.1034/J.1600-0897.2003.01154.X

Steffl, M., Schweiger, M., Sugiyama, T., and Amselgruber, W. M. (2008). Review of apoptotic and non-apoptotic events in non-ciliated cells of the mammalian oviduct. *Ann. Anat.* **190**, 46–52. doi:10.1016/J.AANAT.2007.04.003

Sweet, R. L. (2011). Treatment of acute pelvic inflammatory disease. *Infect. Dis. Obstet. Gynecol.* **2011**, 561909. doi:10.1155/2011/561909

Tuffrey, M., Falder, P., Gale, J., and Taylor-Robinson, D. (1986). Salpingitis in mice induced by human strains of *Chlamydia trachomatis*. *Br. J. Exp. Pathol.* **67**, 605–616.

Vlachos, I. S., Paraskevopoulou, M. D., Karagkouni, D., Georgakilas, G., Vergoulis, T., Kanellos, I., Anastasopoulos, I. L., Maniou, S., Karathanou, K., Kalfakakou, D., Fevgas, A., Dalamagas, T., and Hatzigeorgiou, A. G. (2015). DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic Acids Res.* **43**, D153–D159. doi:10.1093/NAR/GKU1215

Wright, V. C., Chang, J., Jeng, G., and Macaluso, M. (2008). Assisted reproductive technology surveillance – United States, 2005. *MMWR Surveill. Summ.* **57**, 1–23.

Yeruva, L., Myers, G. S., Spencer, N., Creasy, H. H., Adams, N. E., Maurelli, A. T., McChesney, G. R., Cleves, M. A., Ravel, J., Bowlin, A., and Rank, R. G. (2014). Early microRNA expression profile as a prognostic biomarker for the development of pelvic inflammatory disease in a mouse model of chlamydial genital infection. *MBio* **5**, e01241-14.

Zhong, G. (2009). Killing me softly: chlamydial use of proteolysis for evading host defenses. *Trends Microbiol.* **17**, 467–474. doi:10.1016/J.TIM.2009.07.007

Zhong, G. (2011). *Chlamydia trachomatis* secretion of proteases for manipulating host signaling pathways. *Front. Microbiol.* **2**, 14. doi:10.3389/FMICB.2011.00014