Progesterone Activates Multiple Innate Immune Pathways in Chlamydia trachomatis-Infected Endocervical Cells

Progesterone activates multiple innate immune pathways in *Chlamydia trachomatis*-infected endocervical cells

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Abstract:

Problem: Susceptibility to *Chlamydia trachomatis* infection is increased by oral contraceptives and modulated by sex hormones. We therefore sought to determine the effects of female sex hormones on the innate immune response to *C. trachomatis* infection.

Method of study: ECC-1 endometrial cells, pre-treated with estradiol or progesterone, were infected with *C. trachomatis* and the host transcriptome analysed by Illumina Sentrix HumanRef-8 microarray. Primary endocervical epithelial cells, prepared at either the proliferative or secretory phase of the menstrual cycle, were infected with *C. trachomatis* and cytokine gene expression determined by quantitative RT-PCR analysis.

Results: *C. trachomatis* yield from progesterone-primed ECC-1 cells was significantly reduced compared to estradiol-treated cells. Genes up-regulated in progesterone treated and *Chlamydia*-infected cells only included multiple CC and CXC chemokines, IL-17C, IL-29, IL-32, TNFα, DEFB4B, LCN2, S100A7-9, ITGAM, NOD2, JAK1, IL-6ST, type I and II interferon receptors, numerous interferon-stimulated genes and STAT6. CXCL10, CXCL11, CX_3CL1 and IL-17C were also up-regulated in infected secretory stage primary cells and there was a trend towards higher levels of immune mediators in infected secretory phase compared to proliferative phase cells.

Conclusions: Progesterone treatment primes multiple innate immune pathways in hormone-responsive epithelial cells that could potentially increase resistance to chlamydial infection.

Key Words: *Chlamydia*, progesterone, innate immunity, gene array

Introduction:

Chlamydia trachomatis is an obligate, intracellular bacterial pathogen that causes salpingitis, pelvic inflammatory disease, ectopic pregnancies and infertility as well as blinding trachoma. The initial target cells of *C. trachomatis* genital serovars are the epithelial cells of the endocervix in women and the penile urethra in men. Both *in vivo* and *in vitro* studies have shown that the female sex hormones estradiol and progesterone affect susceptibility to infection. For example, oral contraceptive use increases the risk of contracting chlamydial infections [1]. Estradiol treatment of HeLa cells enhances both chlamydial adherence to cells and the development of chlamydial inclusions whist progesterone, in combination with estradiol reduced chlamydial adherence [2]. Using porcine primary genital tract epithelial cells, Wyrick et al. [3] showed that cells isolated at the estrogen-dominant phase of the cycle were more susceptible to infection with *C. suis* than cells isolated from the progesterone dominant stage of cycle. In the mouse model of *C. muridarum* infection, pre-treatment with progesterone is required to achieve infection of 100% of animals. Conversely, guinea pigs are more susceptible to infection after priming with estradiol. Within the female reproductive tract (FRT) the need to protect against sexually transmitted pathogens must be balanced against the need to sustain the development of an allogeneic fetus. Because of this unique requirement many aspects of both innate and adaptive immunity in the FRT are regulated by changes in female sex hormones. These include the expression of chemokine receptors (CXCR4 and CCR5), production of inflammatory cytokines and chemokines (IL-6 and CXCL8/IL-8) and antimicrobial peptides such as SLPI, HBD2 and lactoferrin [4]. Neutrophil and monocyte numbers also fluctuate throughout the reproductive cycle [5] as do the IgG and IgA levels in reproductive tract secretions [4], the numbers of CD8 cells, B lymphocytes and monocytes in endometrial lymphoid aggregates [6], endometrial NK cell numbers [7] and CTL activity of CD8 cells isolated from hysterectomy tissues [8]. Less information is available however on how female sex hormones affect the innate immune response of FRT epithelial cells, the initial cell type targeted by *Chlamydia*. Furthermore, changes in these sex hormones control all aspects of tissue remodelling in preparation for possible fertilization and implantation. Because the effects of the female reproductive hormones are profound, often modulating global gene expression, we have used a gene array approach to investigate how sex hormone treatment of hormone-responsive ECC-1 cells affects the early (12 hours) and late (24 hours) innate immune response to chlamydial infection. Primary endocervical cells

were also isolated from hysterectomy tissues collected at either the secretory or proliferative stage of the menstrual cycle and infected with *C. trachomatis*. Based on changes in gene expression patterns seen in hormone pre-treated and infected ECC-1 cells the expression of selected innate immune response genes was assayed in infected primary epithelial cells using quantitative RT-PCR. Data from both the cell line and primary cell studies show that progesterone activates multiple innate immune pathways that could reduce susceptibility to chlamydial infection.

Materials and Methods:

This study was approved by the Human Research Ethics Committee of the University of Newcastle and Hunter-New England Health. Written informed consent was obtained from all study participants

Cell culture and chlamydial infection

The ECC-1 cell line derived from the human uterine endometrial functional zone [9] was a gift from Dr John Fahey, Department of Physiology, Dartmouth College. ECC-1 cells were cultured in 24-well plates (Nunc) on 10mm diameter coverslips to 80% confluence, before the addition of of 17β-estradiol (200 pg/mL), progesterone (20 ng/mL) (both Sigma-Aldrich) or a combination of both hormones, in phenol red and antibiotic-free DMEM/F12 medium supplemented with 10% charcoal/dextran-treated FBS (Invitrogen), for 24 hrs. The confluent cells (~$5x10^5$ cells) were infected with $5x10^5$ I.F.U. of *C. trachomatis* serovar D (multiplicity of infection [MOI] =1). Plates were centrifuged at 400 x*g* for 45 min at RT, to facilitate infection. After 4 hours cells were washed 3X to remove surface-adherent *Chlamydia* and then incubated for 12 or 24 hrs at 37^0C/5% CO_2.

Isolation of primary human endocervical epithelial cells.

Endocervical tissues were obtained from women undergoing hysterectomy, with an age range of 31-55 years (average age 42.2 years). The stage of the menstrual cycle was determined by a pathologist. Tissues were collected from women undergoing hysterectomy for fibroids, dysmenorrhea and prolapse but not from women with known cancer or a history of abnormal cervical cells. Epithelial cells were isolated as described by Wira et al. [10]. Briefly, the epithelium was separated from underlying stromal tissues by scraping the luminal surface with a scalpel. Epithelial tissues were then minced under sterile conditions into 1-2 mm fragments and digested for 2 hours with an enzyme solution containing pancreatin (3.4 mg/ml, Invitrogen), hyaluronidase (0.1 mg/ml, Sigma-Aldrich) and collagenase (1.6 mg/ml, Sigma-Aldrich) in HBSS supplemented with 2 mg/ml D-glucose. Cells were then dispersed through a 250 μm mesh screen, washed and suspended in phenol red-free DMEM/F12 medium containing 10% charcoal/dextran-stripped FCS (Invitrogen) 20 mM HEPES, 50 U/ml (Invitrogen), Primocin (InvivoGen) and 2 mM L-glutamine (Invitrogen, Complete medium). Epithelial sheets were separated from stromal cells by filtration through a 40 μm mesh, washed by centrifugation and resuspended in

complete medium. Epithelial sheets were aliquoted to 24 well plates pre-coated with Human Extracellular Matrix (BD Biosciences) and cultured in complete medium until confluent (usually 5-7 days). Cells were then rinsed twice with complete medium without Primocin and infected with *C. trachomatis* serovar D or L2 (5×10^6 IFU) by centrifugation (Beckman J-6M/E centrifuge, 1000 rpm for 45 min at 22°C) and then cultured for a further 24 hours. RNA was then prepared by Trizol extraction (Sigma-Aldrich).

Sample preparation for Microarray analysis

At 12 and 24 hours post-infection DNA and mRNA were isolated from triplicate wells of hormone-treated and control ECC-1 cells using the Trizol extraction method according to the manufacturer's instructions. The resultant RNA was resuspended in 10 μL ddH_20 and mRNA purified using the Pure-link RNA extraction kit (Invitrogen), according to the manufacturer's instructions. Separately, the DNA component from the Trizol separation was purified following the manufacturer's instruction. The final eluate for all reactions was collected in a 40 μL volume and quantified using a NanoDrop spectrophotometer (Thermo Scientific) before being used for microarray or RT-PCR analysis.

Illumina Microarray hybridisation and analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and quantified using the Quant-iT RiboGreen RNA Quantitation Assay Kit (Invitrogen), with the fluorescence measured on the FLUOstar Optima (BMG LABTECH) at 485 nm for excitation and 520 nm for emission. A total of 500 ng RNA was reverse transcribed into cRNA and labelled with biotin-uridine triphosphate using the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems). A total of 850 ng cRNA was hybridised to Illumina Sentrix HumanRef-8 v1.1 Expression BeadChips (Illumina) using standard protocols. Each BeadChip measured the expression of 24,354 genes and was scanned using the Illumina Bead Station and captured using BeadScan 3.5.11 (Illumina). For whole-genome gene analysis, data were initially cubic spline normalised using BeadStudio 2.0 software (Illumina), and each individual gene was normalised to the median using GeneSpring GX 11.0, resulting in two-way normalisation.

Genes were judged to be differentially regulated only when: (1) the gene was present in all (triplicate) samples studied, (2) the difference in expression was >1.5-fold, and (3) the extent of difference in expression was significant ($p<0.05$ in Wilcoxon–Mann–Whitney test). The microarray data can be accessed via the Gene Expression Omnibus (GEO) database (GSE31149) and is compliant with MIAME guidelines.

First-strand synthesis of C.trachomatis *D-infected ECC-1 mRNA*

First-strand cDNA synthesis from the 24 hours timepoint was performed with the Superscript III first-strand synthesis kit (Invitrogen) following the manufacturer's instructions. 0.5 mL of each RNA extract was used with oligo-dT to generate host-specific cDNA for comparative analysis.

Quantitative real-time reverse transcriptase PCR analysis

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to confirm the accuracy and expression trends of the microarray data from infected ECC-1 cells and to determine cytokine transcript levels in *C.trachomatis*-infected primary epithelial cells using intron-spanning, gene-specific primers. The primers used in the validation of the microarray data were designed using the software Oligo (Molecular Biology Insights) and confirmed online using NCBI's Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences are provided in **Error! Reference source not found.**~~Table 1~~. Individual PCR reactions, containing 5 μL of the Platinum SYBR super-mix (Invitrogen), 1 μM of each forward and reverse primers (Sigma Aldrich) and 1 μL of template in a final reaction volume of 10 μL were used.

The transcript levels from infected primary endocervical cells were measured using the Taqman Gene Expression Assay (Applied Biosystems) following the manufacturer's protocols. Details of the Taqman gene expression assay used are summarised in **Error! Reference source not found.**~~Table 1~~. QRT-PCR data was collected on the Rotorgene Q (Qiagen) version 1.7.94. A standard curve for each gene was constructed using genomic DNA extracted from the ECC-1 cell line, amplified via conventional PCR and purified via

Pure-link PCR columns (Invitrogen). A 100-fold dilution series was constructed with concentrations ranging from 10^8 to 10^2 copies/mL.

A standardised protocol of an initial incubation at 95^0C for 10 min, followed by a touchdown protocol of 94^0C for 45 sec, 70^0C-60^0C for 15 sec decreasing by 1^0C each cycle, and 72^0C for 20 sec for a total of 10 cycles, followed with 30 cycles of 94^0C for 45 sec, 60^0C for 15 sec and 72^0C for 20 sec was used. Real-time data acquisition for fluorescence was taken upon completion of the extension step. Melt curve analysis was performed between 75^0C and 95^0C at a 1^0C intervals, holding for 5 seconds at each interval to confirm the extension of the correct homogenous product. All samples (cDNA and gDNA) were amplified in triplicates, and then compared to freshly diluted standard curves (amplified in duplicates). Interrogation of each gene product was performed within the same run to eliminate inter-run variability. Quantitation of chlamydial genomic DNA and cDNA of the transcripts was performed using *Chlamydia* specific primers for the chlamydial 16SrRNA ORF. Amplification involved an initial denaturation step at 94^0C for 10 min, then 40 cycles at 94^0C for 30 sec, 50^0C for 15 sec then extension at 72^0C for 30 sec followed by data acquisition at the end of the extension.

Ingenuity Pathway Analysis of up-regulated interferon regulated genes

Ingenuity Pathway Analysis software (version 8.5) was used to examine the degree of global up-regulation mediated by each of the hormone $\pm$ infection conditions. Linkages and associations were made with genes that are up-regulated under progesterone only treatment, and dual hormonal effect. A system default parameter was used in all analysis, to link conical pathways with up-regulated genes, and neighbourhood linkage, unless otherwise stated. The status of each hormone-mediated up-regulated gene from the different treatments was tallied against the Interferome database (http://interferome.org/) to establish whether its regulation is affected by the presence of interferon-gamma (IFNγ) in previously published literature.

Results:

Effect of sex hormone supplementation on C. trachomatis infection of ECC-1 cells.

To determine if sex hormones affected chlamydial infection of ECC-1 cells we measured total chlamydial genomic DNA at 24 hrs post-infection (p.i.) by qPCR, targeting the chlamydial 16S ribosomal RNA (16S rRNA). The number of chlamydial genomes was calculated from the geometric mean of 16S rRNA triplicates of each hormone treatment. After accounting for the fact that *C. trachomatis* has two copies of the 16S rRNA gene, the number of *Chlamydia* in progesterone-treated ECC-1 cells was found to be significantly lower than in the other hormone treatments, as well as the no hormone condition. Analysis using one-way ANOVA indicates a statistically significant reduction in *Chlamydia* between progesterone-only exposure and dual hormone exposure ($p<0.008$), and between the progesterone and no hormone treatment ($p<0.013$, Figure 1). No significant difference between estradiol treatment and progesterone treatment ($p<0.081$) was found,, probably due to the high variance within the estradiol-treated triplicates. In progesterone-treated ECC-1 cells, *C.trachomatis* copy number was almost 10-fold lower than cells treated with both hormones together (Figure 1) indicating that the suppressive effect of progesterone on chlamydial infection is negated by estradiol.

Global gene expression profiling under different hormone treatments.

We then compiled global gene expression profiles for ECC-1 cells treated with hormones alone, or pre-treated with hormones prior to chlamydial infection, at both 12 and 24 hrs, after normalising against similarly treated no hormone and no infection controls. This is summarised in Figure 2. From a total of 22184 genes on the microarray, the number of differentially expressed genes that were significantly changes at 12 hrs (Figure 2A) was 2552 (315 up, 2237 down; **11.5%** of the genome) for estradiol, 2716 (400 up, 2316 down; **12.24%**) for progesterone, and 2638 (342 up, 2296 down; **11.89%**) for both hormones together. At 24 hrs (Figure 2B), the number of genes that were differentially expressed dropped to 1461 (980 up, 481 down; **6.59%**) for estradiol, 585 (385 up, 200 down; **2.64%**) for progesterone, and 2429 (1500 up, 929 down; **10.95%**) for both hormones. When infected with *Chlamydia*, the number of significant differentially expressed genes at 12 hrs. numbered 1871 (1274 up, 597 down; **8.43%**) for estradiol, 1647 (874 up, 773 down;

7.42%) for progesterone, and 2024 (1219 up, 805 down; **9.12%**) for both hormones. At 24 hrs p.i., the number of genes significantly altered changed to 1658 (1090 up, 568 down; **7.47%**), 1842 (1489 up, 353 down; **8.30%**), and 1381 (831 up, 550 down; **6.23%**) for estradiol, progesterone, or both hormones respectively.

Induction of cytokines, chemokines, and immune-related gene expression in the human ECC-1 cells by progesterone alone.

To establish the potential mechanism(s) that limit chlamydial growth in the presence of progesterone, we focused on the immune response genes encoding innate immunity components, signal transducers and transcriptional modulators. Genes significantly up-regulated by progesterone but not estradiol or a combination of both hormones were identified and categorised either as a positive or negative interferon-gamma-response genes (IRG) using the online Interferome database (http://interferome.org) because both type I and type II interferon pathways have been implicated in both clearance of *Chlamydia* and chlamydial inflammation. Table 2 summarises the chemokines, cytokines and immune-related genes that were up-regulated at the 12 and 24 hours in progesterone pre-treated cells plus or minus infection with *C. trachomatis* D

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Progesterone-specific up-regulated genes in non-infected cells at 12 and 24 hrs included two chemokines (CXCL13 [2.6-fold] at 12 hrs and CXCL7/PPBP [3.3-fold] at 24 hrs). Three cytokine genes (IL-2 [1.8-fold], IL-19 [1.5-fold] and IL-24 [3.2-fold] were all increased at 12 hrs. Four immune-related genes (ITFG1 [6-fold] at 12 hrs and DEFB108B [2.7-fold], IL-20RB [3-fold] and SOCS4 [1.5-fold] at 24 hrs) were also increased in progesterone-treated cells. All genes were classified as IRG-, with the exception of CXCL7/PPBP, according to the interferome database.

When progesterone pre-treated ECC-1 cells were infected with *C. trachomatis*, the number of up-regulated transcripts was more pronounced. At 12 hrs., there was an elevated production of β defensins (DEFB104A, DEFB129, DEFB137) as well as the α defensin, DEFA4 and transcripts for the receptors for IL-6 and CXCL8/IL-8 (2- and 3-fold, respectively). Consistent with the observed recruitment of leukocytes into *Chlamydia*-infected tissues, transcripts encoding multiple chemokines, both CC and CXC, were significantly increased at 12 and particularly 24 hours. At 12 hours p.i., the expression of

CCL4 (recruitment of monocytes, NK cells, and dendritic cells) and CXCL12 (recruitment of neutrophils, monocytes, T- and B-cells) were increased by 2.8 and 2.6-fold respectively. Transcripts encoding matrix metalloproteases (MMP3, MMP7 and MMP25) and peptides of the S100 family (S100A2 at 12 and 24 hours and S100A3/7/8/9 at 24 hours) were also detected. Included among the many functions of S100 family proteins are leukocyte chemoattraction, anti-microbial activity and pro-inflammatory functions [11].

After prolonged exposure to the pathogen, there was increased transcription of multiple IRG^+ and IRG^- genes. Notable among these were CXCL8/IL-8 [5.8-fold], CCL20 [8-fold], CXCL2 [6.3-fold], CXCL11 [6.3-fold], and CX_3CL1 [7.6-fold]. Three of these chemokines (CCL20, CXCL2 and CXCL11) possess potent antimicrobial activity. Interestingly, transcript products encoding TNFα [4.42-fold], IL-1β [4.8-fold], IL-6 [4.1-fold], IL-17C [8.1-fold], IL-21 [5.3-fold] and IL-23A/p19 [3-fold] were up-regulated in concert. The gene for IL-29, a type 3 interferon was up-regulated [2.1-fold] at 12 hours and transcripts encoding the pro-inflammatory cytokine IL-32 were increased [2.6-fold] at 24 hours.

One of the variant IL-6ST (gp130) genes, a component of the receptor for the IL-6 family of cytokines, was increased by 11-fold along with the gene for its ligand IL-6. Transcript products associated with signal transduction and transcriptional modulators were found to be up-regulated as well: with JAK1 [6.2-fold], STAT5A [3.8-fold], NOD2 [2.81-fold], STAT6 [2.3-fold], SOCS1 [4-fold] and SOCS4 [1.6-fold] being the most prominent. Increased expression of some cytokine receptors were also detected, with IFNAR1 [2.3-fold], IFNAR2 [2-fold], IFNGR2 [3.5-fold] and IL-18R1 [2.5-fold] transcripts being up-regulated. Various membrane adhesion molecules were also up-regulated: ICAM1 [3.9-fold], ITGAM [2.5-fold], and ITGB8 [3.2-fold] all of which serve a role in intercellular attachment.

Quantitative PCR verification of microarray data.

Using qRT-PCR, five up-regulated genes were randomly chosen (CCL20, CX_3CL1, CXCL8/IL-8, INFAR1 and CXCL11) to verify the accuracy and reliability of our microarray data. Complementary DNA for each gene from respective hormone-treatments were quantified, then normalised against the copy number of their respective genomic counterparts. From the resultant ratio the level of fold change was calculated, comparing infected and non-

infected cell lines exposed to similar hormone conditions. In all cases the trend of up-regulation for the qPCR data mirrors our microarray data (Figure 3).

Increased expression of cytokine and chemokine genes in human secretory phase primary endocervical cells.

Quantitative RT-PCR was used to compare expression levels of selected cytokines/chemokines in primary endocervical cells, collected at either the secretory or proliferative phases of the menstrual cycle (Figure 4). The genes were chosen based on those that were changed following progesterone treatment and infection of ECC-1 cells. Statistically significant differences in the levels of transcription in *C. trachomatis* D infected primary cells, collected at the estradiol dominant proliferative phase compared to the progesterone dominant secretory phase, were seen for CXCL10 ($p<0.0171$), CXCL11 ($p<0.0098$) and IL-17C ($p<0.048$), with the highest expression levels seen in the secretory phase cells. Infection of primary endocervical cells with a second *C. trachomatis,* serovar L_2 also resulted in higher expression of transcripts for CXCL10 ($p<0.0441$) and CXCL11 ($p<0.019$) in secretory phase cells (data not shown). For all of the mediators tested there was a trend towards higher expression of transcripts following infection of cells collected at the secretory phase of cycle.

Discussion:

Chlamydia trachomatis is the most common bacterial sexually transmitted pathogen worldwide and a major cause of pelvic inflammatory disease (PID), ectopic pregnancy and tubal factor infertility. Previous studies have shown that infection rates are lower during the progesterone-dominant secretory phase of the female menstrual cycle. The influence of female sex hormones on chlamydial infectivity is also supported by studies in animal models and in *in vitro* studies using hormone-responsive cell lines. Understanding the mechanism(s) whereby hormones affect susceptibility to infection may provide important insights into understanding and controlling infection-induced pathology and also the development of an effective vaccine. To determine if progesterone modulated innate immune responses to infection, we investigated the global gene expression in hormone-responsive ECC-1 cells, pre-treated with physiological concentrations of progesterone, prior to infection with *C. trachomatis*.

To confirm the suppressive effect of progesterone on intracellular infections, we monitored the genomic copies of *Chlamydia* under the different hormonal conditions, and found a 5-10-fold reduction of *Chlamydia* in ECC-1 cells pre-treated with progesterone. Under the effect of progesterone alone there was also a significant global suppression of gene expression (10.46%) at 12 hours, however estradiol pre-treatment also caused a similar suppression of gene expression (10.08%). While less than 2% of genes were up-regulated by progesterone treatment alone at either 12 or 24 hours, chlamydial infection resulted in an increase in the number of up-regulated genes at both 12 (3.94%) and 24 (6.71%) hours. Increased levels of gene expression, following infection of estradiol-treated cells was less evident, only increasing from 4.42% to 4.91% of total genes.

To define the immune components involved in the reduced number of *C.trachomatis* recovered from progesterone-treated cells, we filtered the list of statistically significant candidate genes that were up-regulated in uninfected progesterone-treated cells, but not in estradiol-treated cells. Prior to infection, there was a significant up-regulation of two chemokine genes (CXCL13 and CXCL7) in progesterone-primed cells compared to estradiol-primed cells. Genes encoding IL-2, IL-19 and IL-24 were also increased, as were the ITFG1, DEFB108B and IL20RB genes. Of these genes only the gene encoding CXCL7 was regulated by interferon (IRG+). Thus, progesterone alone enhances expression of genes

encoding chemokines that recruit and activate neutrophils and have direct antimicrobial activity (CXCL7) and B lymphocytes (CXCL13). Also up-regulated was the β-defensin gene DEFB108B together with genes encoding the IL-10 cytokine family members (IL-19 and IL-24) and a component of one of the receptors used by this cytokine family (IL-20RB). Little is known regarding the functions of IL-19 and IL-24 although both may have roles in antibacterial responses, tissue remodelling, have tumour suppressor activity and are implicated in psoriasis [12]. The gene encoding the T cell immunomodulatory protein ITFG1 was increased 6-fold within 12 hrs of progesterone priming. These data suggest that progesterone, even prior to infection, activates components of both the innate (defensin production and neutrophil recruitment) and adaptive (B cell recruitment, modulation of T cell function) immune responses within ECC-1 cells.

Following infection the transcriptome in progesterone-primed ECC-1 cells was radically different. At 12 hours post infection genes encoding CCL4 and CXCL12 were both up-regulated and at 24 hours post-infection there was coordinate up regulation of multiple CC and CXC chemokines as well as CX_3CL1. Increased gene expression ranged between 1.7 and 8 fold and the chemokines were split equally between those that were regulated by interferons (IRG+) and those that were not (IRG-). Many of these chemokines have broad-spectrum antimicrobial activity against both Gram negative and Gram-positive bacteria [13]. CCL5 also has anti-viral activity against HSV-1 [14] while CXCL9 is active against *Neisseria* [15], both sexually transmitted pathogens. Consistent with their chemokine designation this spectrum of up-regulated chemokine genes would result in the recruitment of most leukocyte subsets to the site of infection including neutrophils (CXCL8/IL-8, CCL20, CXCL2), dendritic cells (CCL2), monocyte/macrophages (CCL2, CXCL10, CX_3CL1), NK cells (CXCL10) and T lymphocytes including Th1 and T memory cells (CCL2, CXCL10, CXCL11, CX_3CL1) [16]. Some of these chemokines also promote adhesion of immune cells to endothelial and epithelial cells (CX_3CL1 and CXCL10) and have been implicated in the *de novo* formation of lymphoid tissues (CCL20) [17]. Interestingly, the activity of many chemokines can be modulated by matrix metalloproteinases (MMPs) [18] and 4 MMP genes were up-regulated more than 2.5-fold in progesterone-primed *Chlamydia*-infected cells. MMPs are also involved in immune cell recruitment and attachment through degradation of extracellular matrix proteins and receptor modulation and also implicated in inflammatory tissue damage [19]. We also used qRT-PCR to look at expression of selected chemokines in

Chlamydia-infected primary human endocervical cells (Figure 4). Two CXC chemokines (CXCL10 and CXCL11), two CC chemokines (CCL2 and CCL20) and CX_3CL1 were all expressed in infected, secretory phase cells at higher levels than in infected proliferative phase cells when estradiol is dominant, supporting our cell line data showing that progesterone treatment primes cells to respond in a more robust manner to infection.

Consistent with previous studies of chlamydial infection, genes encoding IL-1 ・, IL-6, CXCL8/IL-8 and TNFα were all up-regulated in progesterone-primed infected cells, as were components of the IL-6 (IL-6ST/GP130 [>11-fold]) and CXCL8/IL-8 (IL-8RB/CXCR2) receptors. Coordinate expression of these 4 cytokines is a common signature associated with many bacterial infections and inflammatory responses. In addition, two other IL-1 family genes (IL-1F8 and IL-1F9) and several defensin-encoding genes (both α and β) were up-regulated in progesterone-primed infected cells, together with several members of the S100 family of proteins (Calgranulins), which have antimicrobial activity [11] and also regulate inflammation, cell growth and differentiation [20,21].

Genes encoding IL-15, a growth factor for NK cells [22] and IL-21, which is involved in regulation of NK, B and T cell function were increased at 24 hours post infection, with IL-21 expression increased more than 5-fold [23]. Most studies of IL-21 suggest that activated CD4 T cells are the primary source of this cytokine [24] so increased gene expression in endocervical cells was unexpected. Expression of these two cytokines may play a role in the early recruitment of NK cells into the infected genital tract. NK cells provide an important early source of IFN ・, which is essential for resolution of chlamydial infections. IL-21 is also important for the development of Th17 cells [24], which are increasingly implicated in the control of genital chlamydial infections [25,26]. Intriguingly, the cytokine that showed the greatest up-regulation was IL-17C. This is the least-well studied member of the IL-17 family of cytokines [27]. T cells are not a source of this cytokine and mRNA for IL-17C has been previously reported in the prostate, kidney, psoriatic skin and infected lung tissues in mice, although expression may be more restricted in humans [27,28]. IL-17C activates NF-kB and stimulates the secretion of TNFα and IL-1β by THP-1 cells and contributes to the exacerbation of inflammatory arthritis in mice [27, 29]. Interestingly, we also found IL-17C mRNA expression in *Chlamydia*-infected primary endocervical cells (Figure), with expression significantly higher in cells isolated at the secretory phase of the reproductive cycle. This is the first report of expression of IL-17C in the reproductive tract.

Genes for two recently discovered cytokines, IL-29 and IL-32, were up regulated in progesterone-treated infected cells. IL-29 (IFN-λ1) is a member of the type III/λ-interferon family [30]. These cytokines are distantly related to type I interferons, and activate JAK-STAT pathways to induce antiviral (including HSV), anti-proliferative and antitumor immune responses (reviewed in [31]). IL-29/IFN-λ1 may inhibit the development of Th2 responses by inhibiting production of IL-13, IL-4 and IL-5 [32, 33], which could promote the development of protective Th1 immunity to infection. IL-32 activates p38MAPK and NF-kB pathways and is implicated in both innate and adaptive immune responses [34]. IL-32 increases production of TNFα, IL-1β, IL-6 and CXCL2/MIP-2 in a dose-dependent manner and epithelial cells from colon, lung and stomach have been shown to secrete IL-32 [34]. IL-32 also synergizes with NOD-1 and NOD-2 ligands for increased production of the pro-inflammatory cytokines IL-1β and IL-6, through a caspase-1 dependent mechanism [35]. At least 3 species of *Chlamydia* (*C. trachomatis, C. muridarum and C. pneumonia*) activate host inflammatory responses in part through NOD-1 [36-39] suggesting that immune activation by *Chlamydia* may involve IL-32/NOD-1 signalling pathways. Furthermore, siRNA knockdown of IL-32 reduces the secretion of Th1 cytokines in human PBMC [40] suggesting a role for IL-32 in driving Th1 immunity.

Type 1 interferon pathways were also activated in progesterone-primed and infected cells with two alpha interferon family members (IFNA1 and IFNA14) and both chains of the receptor for alpha and beta interferons (IFNAR1 and IFNAR2) significantly increased. Type 1 interferons inhibit chlamydial growth in human epithelial cells in a L-tryptophan-dependent manner [41]. The non-ligand-binding chain of the receptor for IFNγ (IFNGR2) was also up-regulated at 24 hours post-infection. A receptor for IFNγ would be required on infected epithelial cells for induction of IDO-mediated tryptophan depletion, the major anti-chlamydial response in human cells elicited by IFNγ secreted by NK and CD4 T cells [42].

In summary, our studies have shown that priming of endocervical cells with progesterone, at concentrations found during the luteal/secretory phase (20 ng/ml), enhances the innate immune response elicited by *C. trachomatis* infection resulting in a 5-10-fold reduction in IFU compared to non-hormone or estradiol-treated cells. The transcriptional profile in progesterone-treated, infected cells included (i) increased expression of multiple antibacterial mediators (defensins, chemokines, S100 proteins), (ii) increased pro-inflammatory cytokines (IL-1, IL-6, TNF ・) (iii) coordinated expression of mediators

involved in immune cell recruitment (chemokines, adhesion molecules, integrins, MMPs), (iv) activation of both type I and type II interferon pathways and (v) production of cytokines that enhance Th1 immunity (IL-32) and suppress Th2 cells (IL-29). This contrasts with the immunosuppressive effects of progesterone at higher concentrations found during pregnancy (50-200 ng/ml in the third trimester) or produced locally in the placenta, where levels can reach 3000 ng/g of tissue [43], which are required to maintain maternal tolerance to the fetus [44]. At higher doses progesterone suppresses NK cell activity [45], promotes the development of Treg cells [46 47], promotes Th2 development over Th1 [48 48], suppresses dendritic cell function [49 50 51] and suppresses antibody production [5]. Collectively these data suggest that multiple protective anti-chlamydial immune mechanisms are primed by progesterone, at concentrations found during the secretory phase, and that this can explain the reduced susceptibility to infection during the secretory phase.

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Figure legends

Figure 1. Following pre-treatment with sex hormones, *C. trachomatis* 16SrRNA levels in ECC-1 cells were determined by qRT-PCR at 24 hours post infection. The significance of differences in chlamydial genome numbers was determined by one-way ANOVA. Data is presented as mean ± SEM of triplicate cultures from one of 3 separate experiments.

Figure 2. Global transcriptome analysis of ECC-1 cells following treatment with sex hormones and/or C. trachomatis infection

Figure 3. Comparison of the fold change of normalised data obtained from qRT-PCR and microarray for the transcripts encoding CCL20, CX_3CL1, CXCL8/IL-8, IFNAR1 and CXCL11 at 24 hrs post-infection of ECC-1 cells. Data are from one of 3 separate experiments.

Figure 4. The fold-change in cytokine and chemokine gene expression between *C. trachomatis*-infected versus non-infected proliferative (P, n = 10)) and secretory (S, n = 10) phase primary endocervical cells was determined by qRT-PCR. The dotted line represents the mean of the population. Data are presented as the mean ± SEM. The asterisk (*) indicates genes that were statistically different.

650 ***Figures and Tables***

651

652 **Figure 1**

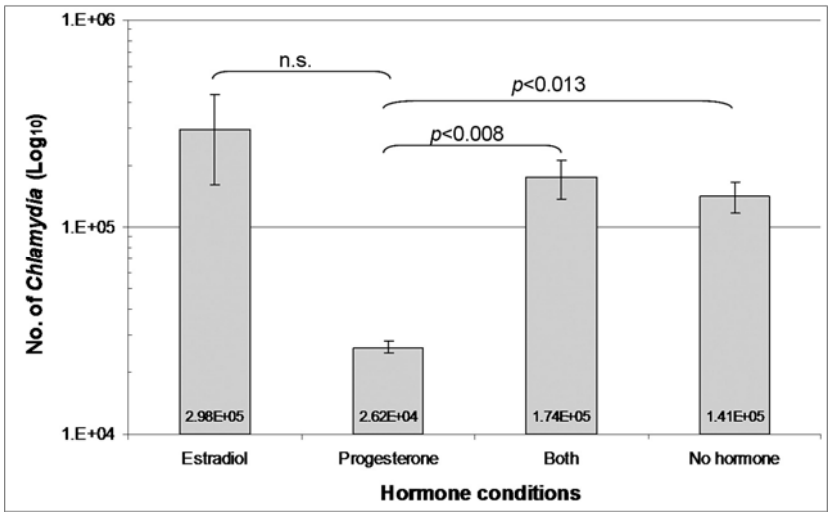

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Figure 2A

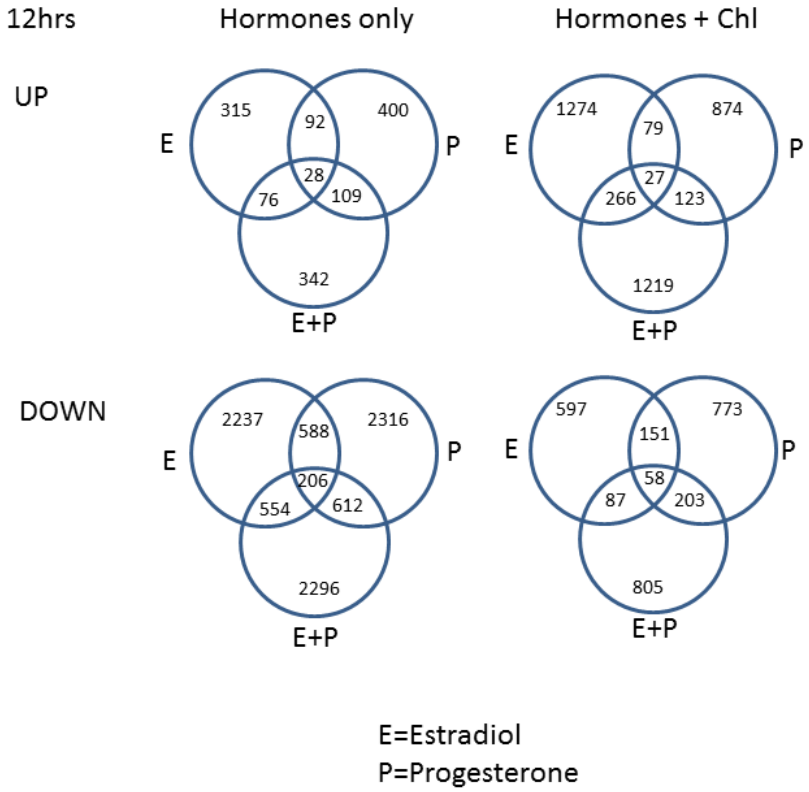

12hrs
Hormones only
Hormones + Chl
UP
E
P
E+P
315
92
400
28
76
109
342
1274
79
874
27
266
123
1219
DOWN
2237
588
2316
206
554
612
2296
597
151
773
58
87
203
805
E=Estradiol
P=Progesterone

Figure 2B

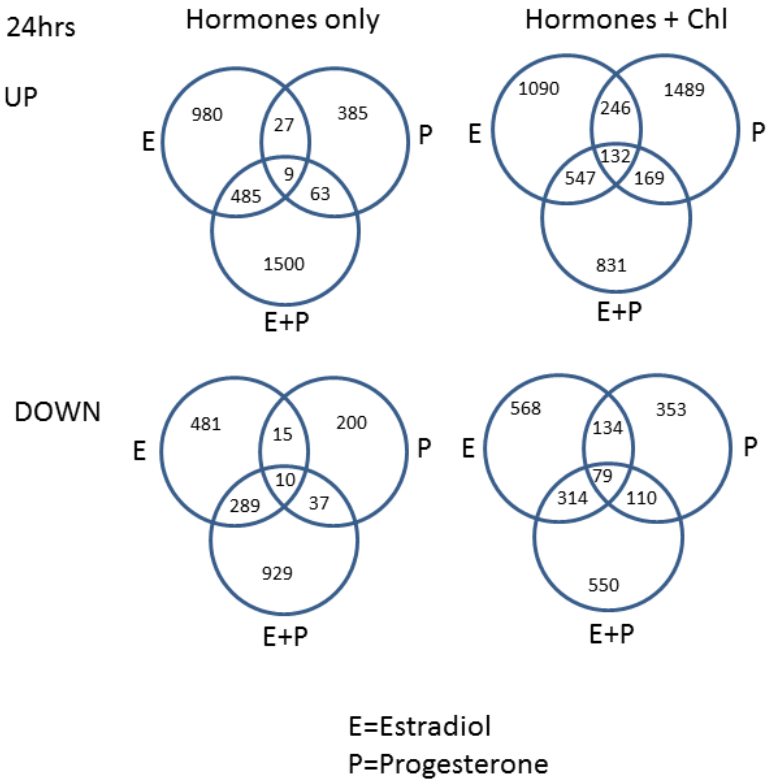
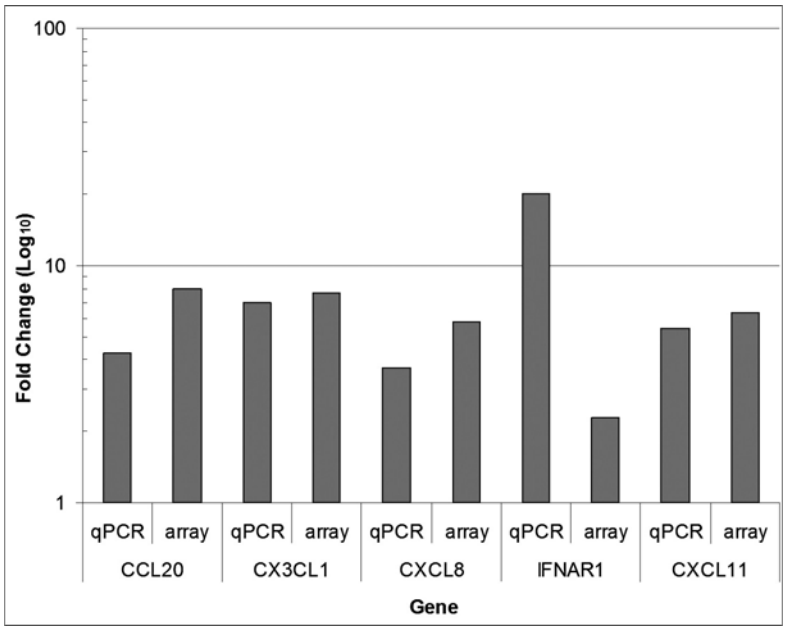

24hrs
Hormones only
Hormones + Chl
UP
980
27
385
E
P
9
485
63
1500
E+P
1090
246
1489
E
P
132
547
169
831
E+P
DOWN
481
15
200
E
P
10
289
37
929
E+P
568
134
353
E
P
79
314
110
550
E+P
E=Estradiol
P=Progesterone

Figure 3


100
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1
Fold Change (Log10)
qPCR
array
qPCR
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qPCR
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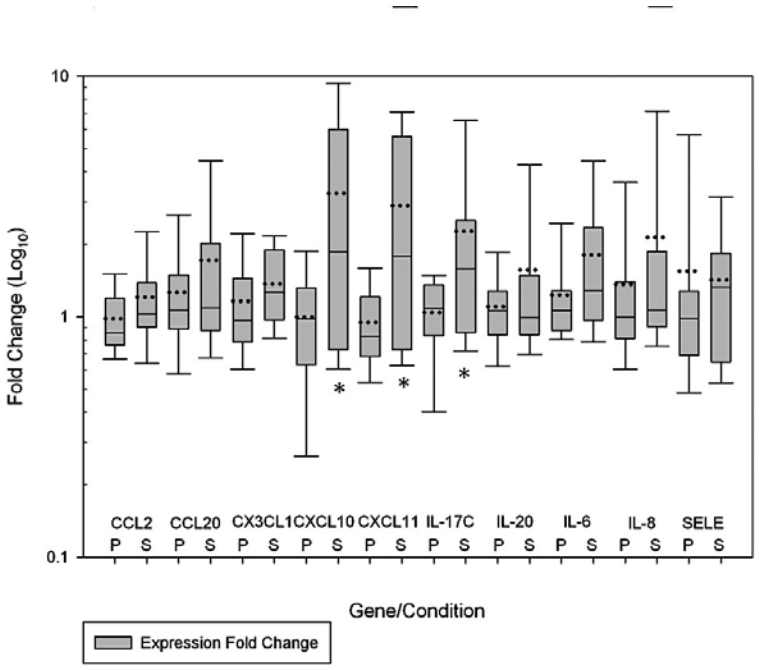
CCL20
CX3CL1
CXCL8
IFNAR1
CXCL11
Gene

669 **Figure 4**

670



671

672 **Table 1.** Gene targets and primer sequences used to quantify: **A**. Microarray data, and **B**.
673 Explanted and infected endocervical tissue

A.

Gene	Accession No.	Sense	Antisense
CCL20	D86955	5'-TAACAGCACTCCCAAAGAAC-3'	5'-GTAGCAGCACTGACATCAAA-3'
CX_3CL1	U84487	5'-CTTGACCATTCTCCACCTTC-3'	5'-CTCTAACCAGCCAGCAGCAG-3'
CXCL8/ IL-8	Y00787	5'-CAAACTTTCAGAGACAGCAG-3'	5'-ACACAGTGAGATGGTTCCTT-3'
IFNAR1	P17181	5'-GTGCAGAGGGGCGGTGTGAC-3'	5'-ACACAGTGAGATGGTTCCTT-3'
CXCL11	U66096	5'- AGTCTTCCTGAATGAATGAC-3'	5'-CTTTGTAAACTCCGATGGTA-3'
*C.tr*D 16S rRNA	D85721	5'-GCTTGTTGGTGGGGTAA-3'	5'-CAGTGTTGGCGGTCAAT-3'

674

B.

Gene	Accession No.	Taqman Gene Expression Assay number
CX3CL1	NM_002996.3	Hs00171086_m1
CXCL8/IL-8	NM_000584.2	Hs00174103_m1
CCL2	NM_002982.3	Hs00234140_m1
CCL20	NM_004591.1	Hs00355476_m1
IL-6	NM_000600.1	Hs00174131_m1
CXCL10	NM_001565.2	Hs00171042_m1
IL-17C	NM_013278.3	Hs00171163_m1
CXCL11	NM_005409.3	Hs00171138_m1
IL-20	NM_018724.3	Hs00218888_m1
Selectin E	NM_000450.1	Hs00174057_m1

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678 **Table 2.** Host-specific immune genes compiled from the microarray data that were
679 significantly up-regulated, in response to progesterone-only treatment, sampled at 12 hrs
680 and 24 hrs time-points, with or without a concurrent *C.trachomatis* D infection.

		No chlamydial infection						Concurrent chlamydial infection					
		12hr			24hrs			12hr			24hrs		
		Name	p-value	fold-Δ	Name	p-value	fold-Δ	Name	p-value	fold-Δ	Name	p-value	fold-Δ
Chemokine	IRG+				PPBP	0.022	3.33				CCL20	0.000	8.00
											CX3CL1	0.000	7.65
											CXCL2	0.001	6.25
											CXCL10	0.000	5.22
											CCL5	0.000	3.81
											CXCL9	0.039	1.70
	IRG-	CXCL13	0.015	2.60				CCL4	0.030	2.80	CXCL11	0.001	6.32
								CXCL12	0.025	2.61	CCL2	0.001	5.56
											CCL22	0.001	5.18
											CCL17	0.008	3.83
											CCL4L2	0.008	2.99
											CXCL1	0.034	2.30
											CXCL6	0.015	2.00
Cytokine	IRG+										IL1B	0.001	4.83
											IL6	0.000	4.10
											IL7	0.007	2.02
	IRG-	IL24	0.030	3.17				IL29	0.042	2.11	IL17C	0.000	8.10
		IL2	0.034	1.76							IL20	0.000	6.23
		IL19	0.035	1.52							IL8	0.000	5.75
											IL21	0.025	5.28
											IL23A	0.000	2.95
											IL32	0.001	2.57
											IL15	0.005	2.00
Immune-related	IRG+							MMP12	0.028	1.56	IL6ST	0.033	11.20
											JAK1	0.038	6.24
											S100A3	0.007	4.20
											SOCS1	0.002	4.07
											ICAM1	0.000	3.94
											IFNGR2	0.000	3.49
											MMP9	0.043	3.12
											ITGAM	0.007	2.50
											TLR5	0.039	2.34
											IFNAR1	0.001	2.30
											STAT6	0.017	2.27
	IRG-	ITFG1	0.023	6.01	IL20RB	0.036	2.94	DEFB129	0.001	6.08	S100A9	0.000	10.22
					DEFB108B	0.005	2.66	IFNA1	0.024	4.41	DEFB4B	0.000	7.42
					SOCS4	0.030	1.52	IFNA1	0.024	4.41	S100A8	0.000	4.90
								DEFA4	0.044	3.98	IL1F9	0.001	4.25
								TLR8	0.035	3.75	STAT5A	0.002	3.80
								MMP25	0.007	3.70	MMP25	0.007	3.53
								DEFB104A	0.007	3.17	ITGB8	0.002	3.22
								IL8RB	0.029	2.95	IL1RAPL1	0.023	2.85
								S100A5	0.021	2.66	S100A7	0.000	2.55
								MMP3	0.038	2.53	IL18R1	0.003	2.44
								MMP7	0.036	2.50	IFNAR2	0.009	2.05
								S100A2	0.018	2.33	ITFG2	0.023	1.68
								DEFB137	0.030	2.32	SOCS4	0.049	1.63
								IL1F8	0.004	1.90			
								IL6R	0.049	1.84			
								IFNA14	0.048	1.69			

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