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Innate immune mediator profiles and their regulation in a novel polarized immortalized epithelial cell model derived from human endocervix

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Abstract

The endocervix in the female reproductive tract (FRT) is susceptible to sexually transmitted pathogens such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. Endocervical epithelial cells *in vivo* make innate immune mediators that likely aid in the protection from these pathogens. *In vitro* studies to investigate the innate epithelial cell immune response to endocervical pathogens have been hindered by the paucity of human endocervix-derived epithelial cell lines that display the differentiation proteins and functional characteristics of their site of origin. We have established an immortalized epithelial cell line (A2EN) derived from an endocervical tissue explant that can be polarized to exhibit distinct apical and basolateral membrane domains. Polarized A2EN cells secrete mucus at their apical surface, and express MUC5B, a mucin specific to the endocervix. Polarized A2EN cells also express hormone receptors that respond appropriately to female steroid hormones. Polarized A2EN cells can be stimulated with the toll-like receptor 3 agonist, polyI:C, to express anti-microbial peptides (AMPs) as well as pro-inflammatory cytokines and chemokines. Cytokines and chemokines are also differentially secreted depending on the hormone milieu in which the cells are exposed. We conclude that polarized A2EN cells maintain distinctive phenotypic and functional characteristics of the

epithelial cells found in the endocervix and, hence, could provide a useful, new *in vitro* model system for investigations on the role of endogenous and exogenous factors that regulate endocervical epithelial cell immunity including studies on sexually transmitted infections and topical microbicides.

Keywords

Anti-microbial peptide; Cytokine; Female genital tract; Hormone

1. Introduction

The endocervix has been described as the ‘Colossus of Rhodes’ of the FRT as it is located between the microbe-rich lower tract and the relatively sterile upper tract (Quayle, 2002). The endocervix, therefore, is likely an important site involved in both innate and adaptive immune responses that participate in the delicate balance between tolerance necessary for conception and protection from pathogens. The endocervical epithelium in the FRT forms crypts or pseudoglands that are lined by mucus-secreting simple columnar cells. Innate immune functions contributed by the epithelium in the endocervix include: (i) provision of a physical barrier through the formation of tight junctions; (ii) production of anti-microbial peptides (AMPs); (iii) production of mucus that traps commensal and pathogenic organisms; (iv) the expression of toll-like receptors (TLRs), receptors that when bound by pathogen-associated molecular patterns (PAMPs), signal the cells to secrete cytokines/chemokines in order to recruit and regulate the activity of leukocytes; and (v) expression of the polymeric immunoglobulin receptor, a molecule that mediates transepithelial transport of polymeric immunoglobulins (Gipson, 2005; Herbst-Kralovetz et al., 2008; Hickey et al., 2011; Quayle, 2002; Wira et al., 2010).

The endocervix is the most common site of infection by *Chlamydia trachomatis*, an obligate intracellular pathogen that is also the most prevalent sexually transmitted bacteria. The pathology associated with this infection has been hypothesized to be initiated and sustained by the epithelial cytokine and chemokine response induced by *C. trachomatis* and, hence, is an important area to be studied (Stephens, 2003). *Neisseria gonorrhoeae*, *Mycoplasma genitalium* and some subtypes of HPV also infect the endocervix, and primate studies have also suggested that this tissue may be an important site of transmission and/or a reservoir for the human immunodeficiency virus (HIV) (Anderson et al., 2010; Belinson et al., 2010; Falk et al., 2005; Haase, 2011; Brunham et al., 1984; Zhang et al., 1999). While a number of *in vitro* studies of genital tract infections have been reported using primary ectocervical and endometrial epithelial cells, few studies have examined pathogen–host interactions in primary endocervical cells. This is likely due to difficulties in growing sufficient quantities of primary epithelial cells from the endocervix because of its relatively small surface area. Limitations to establishing appropriate *in vitro* endocervix-derived epithelial cell models are also likely compounded by more generic issues that can arise from the passage of primary and transformed epithelial cell lines derived from FRT tissues, including loss or change in hormone receptor expression, hormone responsiveness, and anti-microbial molecule expression (Isaka et al., 2003; Joly et al., 2009).

Elegant studies by Wyrick’s group utilizing polarized endometrial epithelial cells for *C. trachomatis* studies found major differences in chlamydial infectivity, entry and exit, developmental cycle duration, reactivity to hormones, and reactivity to antibiotics compared with chlamydiae grown in cells cultured on a plastic surface (reviewed in Wyrick, 2006). These studies illustrate the importance of and preference for polarized epithelial cell culture systems that maintain distinct apical and basolateral surfaces in order to better simulate *in*

vivo relationships between the host and relevant infectious organisms. Other areas of research would also benefit from a polarized epithelial cell model that exhibits many of the phenotypic and functional characteristics of endocervical epithelium. These areas include investigations into the endogenous and exogenous factors that modulate epithelial innate immunity, such as female sex hormones, seminal plasma, and topical microbicides.

2. Materials and methods

2.1. Cell culture methods

The A2EN human epithelial cell line was generated in our laboratory from primary epithelial cells grown from endocervical explant tissue as previously described (Herbst-Kralovetz et al., 2008). A2EN cells tested negative for all *Mycoplasma* spp. as determined using the MycoSensor™ QPCR Assay Kit (Agilent Technologies) according to the manufacturer's instructions. A2EN cells were grown in a phenol red-free, serum-free medium (EpiLife®; Cascade Biologics) with EpiLife® Defined Growth Supplement (Cascade Biologics). The epithelial cells were grown in an atmosphere of 5% CO₂ at 37 °C and experiments were performed on cells grown between passages 17 and 21. To achieve polarization, 2.5×10^4 cells were seeded onto human placental-derived extracellular matrix-coated (BD Discovery Lab) 6.5-mm, 0.4-μm polyester transwell inserts (Corning). After 24 h, apical medium was removed, and basolateral cell culture medium was replaced with differentiation medium containing 0.4 mM Ca⁺⁺. Higher concentrations of calcium have been demonstrated to be necessary for differentiation and tight junction formation to occur in epithelial cell cultures (Hennings et al., 1980; Zhu et al., 2004). Trans-epithelial electrical resistance (TEER) was measured every 24 h with a voltohmmeter (World Precision Instr.) for up to 12 days. Cells were considered polarized when their TEERs were $> 1000 \Omega/\text{cm}^2$ (Fahey et al., 2005; Halldorsson et al., 2007; MacDonald et al., 2007). Polarized A2EN cell monolayers on membranes with TEER measurements of $>1000 \Omega/\text{cm}^2$ were embedded in paraffin, and paraffin sections were then stained with hematoxylin and eosin. Polarized A2EN cells were also fixed, processed, and imaged for transmission electron microscopy as previously described (Beatty, 2006). For all subsequent experiments, polarized A2EN cells were used when their TEERs exceeded $1000 \Omega/\text{cm}^2$, which usually occurred between 7 and 9 days post-differentiation.

Human primary endocervical epithelial cell cultures were also established from endocervical tissue explants obtained from 3 women undergoing hysterectomies for benign gynecological conditions under a protocol approved by the LSU Health Sciences Center Institutional Review Board as previously described (Herbst-Kralovetz et al., 2008; Wang et al., 2011). Primary endocervical epithelial cells from 3 patients were tested for HPV using polymerase chain reaction techniques as previously described (Soler et al., 1991). Primary epithelial cells were polarized under the same conditions as A2EN cells.

2.2. Immunofluorescence staining

Polarized A2EN cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by permeabilization with 0.1% Triton X in PBS for 10 min. Fixed cells were then blocked with Background Sniper (Biocare) for 30 min at room temperature. Cultures to be stained for claudin-1 were incubated with mouse monoclonal anti-claudin-4 clone 3E2C1 (Zymed, Invitrogen, 1:200). Cultures to be stained for MUC5B were incubated with neuraminidase to remove sialic acid residues for 30 min at 37 °C and were then incubated with a mouse monoclonal anti-MUC5B antibody clone 19.4E (AbCam, 1:75). Cultures to be stained for estrogen receptor α (ERα) or estrogen receptor β (ERβ) were incubated with a primary mouse monoclonal antibody (ERα) (Vector Labs, 1:300) or (ERβ) (DAKO, 1:50). For all stainings, primary antibodies were diluted in Davinci Green (Biocare)

antibody diluent and incubated overnight at 4 °C. Cells were then washed with PBS, and goat anti-mouse secondary antibody conjugated to Alexa 488 (Mol. Probes, 1:500) in PBS was added for 30 min at room temperature, followed by washing with PBS. Cells were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Mol. Probes) for 1 min at room temperature followed by washing with PBS. Membranes were excised and mounted using ProLong Gold Mounting Media (Mol. Probes).

2.3. Female sex steroid hormone assays

2.3.1. Estradiol—For studies using estradiol alone, polarized A2EN cells were exposed to culture medium containing 10^{-8} M 17β estradiol in both apical and basolateral chambers for 24 h. For studies using progesterone, polarized A2EN cells were first primed with 10^{-8} M estradiol in culture medium for 24 h. Apical and basolateral culture medium was then replaced with fresh medium containing 10^{-7} M progesterone combined with 10^{-9} M estradiol. All hormone concentrations and protocols were chosen based upon hormone receptor expression studies performed *in vitro* in endometrial epithelial cells (Hombach-Klonisch et al., 2005), and are within the concentration range reported to be found in endometrial tissue (Nussey and Whitehead, 2001).

2.3.2. Tamoxifen—For tamoxifen studies, apical and basolateral surfaces of polarized A2EN cells were exposed to either PBS or Tamoxifen (Sigma) (10^{-5} M, 10^{-4} M, and 10^{-3} M) for 2 h at 37 °C. Treatment was removed, and cells were washed with PBS three times. 10^{-8} M 17β estradiol was added to apical and basolateral chambers for 24 h at 37 °C, after which polarized A2EN cells were fixed, permeabilized, blocked, and stained for ER α and ER β as described in Section 2.2.

2.4. Microscopy and imaging

Fluorescent images were captured using a Leica DMRXA automated upright epifluorescence microscope (Leica Microsystems, Bannockburn, IL, USA); a Sensicam QE charge-coupled device (Cooke Corp., Auburn Hills, MI, USA); and filter sets optimized for Alexa 488 (exciter HQ480/20, dichroic Q495LP, and emitter HQ510/20m) and 4',6-diamidino-2-phenylindole (exciter 360/40 $\times$, dichroic 400DCLP, and emitter GG420LP). Z-axis plane capture, deconvolution, and analysis were performed with SlidebookTM deconvolution software (Intelligent Imaging Innovations, Denver, CO, USA).

2.5. Reverse transcriptase polymerase chain reaction for MUC5B

RNA from polarized A2EN cells was extracted using the Masterpure (Epicentre) RNA extraction protocol according to the manufacturer's instructions. RNA was quantified using the NanoQuant system (Tecan). cDNA was generated from 1 μ g RNA using the Superscript II First Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed for MUC5B detection as previously described (Gipson et al., 1999). PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide, and imaged using the ChemiDoc XRS and Quantity One 4.6.1 software (Biorad).

2.6. Real-time PCR assays

2.6.1. Hormone receptor mRNA quantification—Polarized A2EN cells were either exposed to medium alone, estradiol, or progesterone/estradiol for 24 h as described in Section 2.3.1. HeLa 229 cells grown in RPMI (Gibco) with 10% fetal bovine serum (Gibco) were used as a negative control for hormone receptor expression, and ECC-1 cells grown in DMEM with 10% fetal bovine serum were used as a positive control for hormone receptor expression (Guseva et al., 2005; Mo et al., 2006). Total RNA was extracted and used (2 μ g) to generate cDNA as described in Section 2.5. Quantitative PCR analysis was performed on

an ABI Prism 7500 system (Applied Biosystems, Foster City, CA, USA) to evaluate the relative gene expression of PGR A/B, ESR-1 and ESR-2. The mixture containing sample cDNA (40 ng), specific primer sets (4 pmol), and PCR premix (Power SYBR Green PCR Master Mix, Applied Biosystems) was added to each well on a 96-well reaction plate. All PCR were carried out in triplicate. The expression of β -actin was analyzed in each sample for the normalization of total RNA levels among samples. The primer sequences used were: PGR isoforms A/B (NM_000926) sense 5'-TGG AAG AAA TGA CTG CAT CG-3', antisense 5'-TAG GGCTTG GCTTTC ATTTG-3'; β -actin (NM_001101.2) sense 5'-CAT GTA CGT TGC TAT CCA GGC-3' antisense 5'-CTC CTT AAT GTC ACG CAC GAT-3'; ESR-1 (NM_000125) sense 5'-AGA TCT TCG ACA TGC TGC TGG CTA-3', antisense 5'-AGA CTT CAG GGT GCT GGA CAG AAA-3'; and ESR2 (NM_001437) sense 5'-TCG GAA GTG TTA CGA AGT GGG AAT GG-3', antisense 5'-GCA CTT CTC TGT CTC CGC ACA A-3'. The PCR protocol consisted of: 50°C for 2 min (UDG incubation), 95 °C for 2 min (denaturation), and 45 cycles of: 95 °C for 15 s, 60 °C for 60 s, followed by melt temperature analysis. The PCR products, inserted into the pCRII-TOPO plasmid vector, were confirmed using standard sequence analysis.

2.6.2. TLR mRNA quantification—TLR expression was determined using a reverse transcription real-time PCR protocol for TLRs 1–9 and CD14 as previously described (Herbst-Kralovetz et al., 2008).

2.6.3. Anti-microbial peptide mRNA quantification—Total RNA was extracted, and used (2 μ g) to generate cDNA as described in Section 2.5. Semi-quantitative PCR for the levels of LL37, SLPI, HBD1, HBD2, HBD3, HD5, and HD6 mRNA was performed on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Each PCR contained RT products of 10 ng total RNA equivalent for LL37, SLPI, and HBD2, or 20 ng RNA equivalent for HBD1, HBD3, HD5, and HD6; each primer at 0.1 μ M, and SYBR Green Master Mix (Qiagen). The primer sequences used were as follows:

Table 1

Gene	Forward	Reverse
LL37	5'-GAAGACCCAAAGGAATGGCC-3'	5'-TCAGAGCCCAAGCCTGAG-3'
SLPI	5'-GCATCAATGCCTGGATCCT-3'	5'-AGTCTCAGGGTGAAAGG-3'
HBD1	5'-TTGTCTGAGATGGCCTCAGGTGGTAAC-3'	5'-ATACTTCAAAAGCAATTTTCCTTTAT-3'
HBD2	5'-CCAGCCATCAGCCATGAGGGT-3'	5'-GGAGCCCTTCTGAATCCGCA-3'
HBD3	5'-AGCCTAGCAGCTATGAGGATC-3'	5'-CTTCTTCGGCAGCATTTTC-3'
HD5	5'-AGGTGACACTATAGAATAACCTCAGGTTCTC AGGCAAG-3'	5'-GTACGACTCACTATAGGGATGAATCTTGCACTGCTTTGG-3'
HD6	5'-AGGTGACACTATAGAATATTCATTGCCATT GCAGAAG-3'	5'-GTACGACTCACTATAGGGAATGGCAATGTATGGGACACA-3'
18srRNA	5'-AGGTGACACTATAGAATAGTGAGCGATTGTCTGGTT-3'	5'-GTACGACTCACTATAGGGACGCTGAGC CAGTCAGTGTAG-3'

The PCR cycling conditions included a 95 °C denaturation for 10 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72°C for 30 s. Expression levels of different AMPs were normalized to the level of constitutively expressed 18S rRNA. Expression of all transcripts for each treatment relative to the control were calculated by the $\Delta\Delta C_t$ (Yuan et al., 2006) (C_t , threshold cycle of realtime PCR) method according to the following formulas: $\Delta C_t = C_{t18S\ rRNA} - C_{tAMP}$, $\Delta\Delta C_t = \Delta C_{t\ control} - \Delta C_{t\ treatment}$, $ratio = 2^{-\Delta\Delta C_t}$.

2.7. Cytokine and chemokine multiplex assays

Polarized A2EN cells were washed and exposed to the TLR 3 agonist, polyI:C (100 μ g/mL, Invivogen) in culture medium for 24 h. For all cytokine and chemokine assays, culture

medium with or without treatment was added to the apical chamber after TEER measurements exceeded $1000 \Omega/\text{cm}^2$. Supernatants were then collected at the appropriate time points. Initially, 28 different cytokines and chemokines were quantified from apical and basolateral supernatants using a multiplex cytometric bead array (27 cytokines were measured using the BioPlex Human 27-plex Assay [Biorad], IL1 α was measured using a Milliplex Assay [Millipore]). Only those cytokines and chemokines from this panel that were secreted by A2EN cells were chosen for further studies. These were G-CSF, GM-CSF, IL1 α , IP10, MIP1 β , RANTES, TNF α , IL6, and CXCL8 (Millipore). Concentrations of these cytokines and chemokines were measured in triplicate supernatants collected from both apical and basolateral surfaces. For quantification, duplicate standards produced a curve for each analyte from which concentrations were extrapolated. Medium alone was used to establish background levels of cytokines and chemokines. Unstimulated polarized A2EN cell culture supernatants established baseline cytokine and chemokine secretion.

2.8. Hormonal regulation of cytokine and chemokine secretion

Polarized A2EN cells were exposed to medium alone, estradiol or combined progesterone/estradiol for 24 h as described in Section 2.3.1. Hormone exposed cells were then stimulated with polyI:C as described in Section 2.7 for 24 h. Apical and basolateral supernatants from each sample set were collected and cytokines and chemokines measured as described in Section 2.7.

2.9. Statistical analyses

Non-parametric statistical analyses were completed using Prism software (v4.0; GraphPad, San Diego, CA, USA). One-way analyses of variance (ANOVA) with Bonferroni post-tests were employed. A *p* value of <0.05 was considered significant.

3. Results

3.1. A2EN cells polarize and express the gel forming mucin MUC5B

The human endocervical epithelial cell line A2EN was previously generated in our laboratory from primary epithelial cells grown out from an endocervical tissue explant and immortalized by retroviral transduction with the HPV E6/E7 oncogenes (Herbst-Kralovetz et al., 2008). Similar to previous observations of primary and immortalized epithelial cells derived from endocervical explants, we observed that A2EN cells cultured on a traditional plastic surface predominantly exhibited a keratinocyte-like appearance, with occasional large vesiculated cells (Espinosa et al., 2002; Herbst-Kralovetz et al., 2008). When A2EN cells were grown at an air interface on filter inserts under differentiation conditions, they exhibited contact inhibition and a “cobblestone-like” appearance. A2EN cells grown on filter inserts formed largely single cell monolayers (Fig. 1a) that consisted of cuboidal cells with occasional multilayer regions. After 3–4 days in culture under differentiation conditions, basolateral medium no longer diffused through to the apical surface, creating a dry air interface. Electron microscopy revealed the presence of apical junctional complexes (Fig. 1b and c) that created distinct apical and basolateral domains; this establishment of distinct membrane domains defines polarization (Bryant and Mostov, 2008). Microvilli on the apical surface of polarized A2EN cells were also visible using electron microscopy (Fig. 1b). Fluorescent microscopy revealed a “honey-combed” staining pattern of claudin-4 (Fig. 1d), a protein within the tight junction complex, suggesting that A2EN cells cultured on transwell inserts under differentiation conditions form tight junctions. The functional integrity of A2EN cell monolayers was assessed by transepithelial electrical resistance (TEER) measurements. TEERs were determined from day 1 to day 12 of cultivation under differentiation conditions (Fig. 1e). A time-dependent increase in resistance was observed, indicating continuous cell–cell associations (Fig. 1e). TEERs exceeded $1000 \Omega/\text{cm}^2$ within

7–9 days of culture (Fig. 1e), which is typical of tight junction formation in polarized airway epithelial cell models and primary polarized FRT epithelial cell models (Bryant and Mostov, 2008; Fahey et al., 2005; Klein and Mlodzik, 2005; MacDonald et al., 2007; Wiszniewski et al., 2006). Polarized A2EN cells remained viable for up to three weeks and were capable of maintaining elevated TEER values over this time period.

After 4–5 days under differentiation conditions, A2EN cells grown at an air interface displayed small, circumscribed mounds of material at their apical surface when viewed by inverted light microscopy. Mucin 5B (MUC5B) has been reported to be the most abundant gel-forming mucin in endocervical epithelial cells, and it has been documented to be exclusively expressed in the endocervix in the FRT (Gipson et al., 2001, 1999). We therefore examined MUC5B mRNA and protein expression in polarized A2EN cells. RT-PCR revealed that polarized A2EN cells express MUC5B (Fig. 2a), which was confirmed by sequencing of the RT-PCR product (data not shown). Neither A2EN cells nor HeLa cells cultured on traditional plastic surfaces expressed detectable MUC5B (Fig. 2a), suggesting that polarization of A2EN cells was required for expression of this molecule. MUC5B was also detected at the apical surface of polarized A2EN cells by immunostaining using a specific monoclonal antibody (Fig. 2b), suggesting this was a constituent of the surface material we observed at the apical membrane of the polarized A2EN cultures.

3.2. A2EN cells express functional hormone receptors when polarized

When estrogen binds to either ER α or ER β , the receptor complexes translocate to the nucleus where they modify gene transcription (Beagley and Gockel, 2003; Lea and Sandra, 2007). ER α and ER β were localized to the nucleus in polarized A2EN cells upon exposure to 10^{-8} M estradiol (Fig. 3a, ER β not shown). Without estradiol exposure, neither ER α nor ER β could be visualized in the nucleus of polarized A2EN cells (data not shown). In order to determine whether ER α and ER β were functional, cells were exposed to tamoxifen, an estrogen receptor antagonist, prior to estradiol exposure. Nuclear localization of ER α and ER β was reduced in polarized A2EN cells exposed to tamoxifen in a dose-dependent manner (Fig. 3a), suggesting that both receptors were functional (ER β data not shown). To further examine whether polarized A2EN cells respond appropriately to steroid hormones, we examined transcription of the progesterone receptor gene (PGR), which is altered in response to estradiol exposure (Hombach-Klonisch et al., 2005). Polarized A2EN cells were exposed to 10^{-8} M estradiol, or 10^{-7} M progesterone combined with 10^{-9} M estradiol for 24 h. These concentrations are believed to be representative of the hormone milieu during the proliferative and secretory stages of the menstrual cycle respectively (Hombach-Klonisch et al., 2005). Expression of PGR in polarized A2EN cells was compared with expression levels in non-polarized A2EN cells cultured on a traditional plastic surface. PGR transcripts were significantly up-regulated in polarized A2EN cells upon estradiol exposure (Fig. 3b, $p < 0.001$), which is consistent with studies performed on hormone receptor regulation in endometrial epithelial cells (Hombach-Klonisch et al., 2005). PGR transcripts were down-regulated ($p < 0.001$) in polarized A2EN cells upon combined progesterone/estradiol exposure (Fig. 3b), which is also consistent with endometrial epithelial cell studies (Hombach-Klonisch et al., 2005). Non-polarized A2EN cultures showed an increase in PGR expression ($p < 0.001$) upon exposure to estradiol, but the magnitude of the transcript levels was low compared with those observed in polarized A2EN cells (Fig. 3b). We also examined mRNA expression of ESR-1, the gene for ER α and ESR-2, the gene for ER β under either estradiol or progesterone/estradiol exposure in polarized A2EN cells. Unexposed polarized A2EN cells expressed significantly higher levels of ESR-1 ($p < 0.001$) and ESR-2 ($p < 0.01$) compared with non-polarized cells (Supp. Fig. 1). Estradiol had no effect on either ESR-1 or ESR-2 expression (Supp. Fig. 1). Progesterone/estradiol exposure decreased ESR-1 expression, but increased ESR-2 expression (Supp. Fig. 1). Taken

together, our results indicate that polarized A2EN cells are responsive to female sex steroid hormones.

3.3. Expression of toll-like receptors in polarized A2EN cells

The TLR expression profile (TLR1–9 and CD 14) of polarized A2EN cells was examined using quantitative PCR as we previously described for primary and immortalized epithelial cells derived from endocervical explants and were cultured on traditional plastic surfaces (Herbst-Kralovetz et al., 2008). Polarized A2EN cells expressed high levels of TLR 2, 3, 4, 5 and 6 and low levels of TLR 1 mRNA (Supp. Fig. 2). TLRs 7–9 were not expressed in these cells (Supp. Fig. 2). These results were consistent with our previous publication, indicating a similar TLR profile in polarized A2EN cells and non-polarized A2EN cells cultured on a traditional plastic surface (Herbst-Kralovetz et al., 2008).

3.4. PolyI:C stimulates anti-microbial peptide expression in polarized A2EN cells

The anti-microbial peptides HBD1, HD5, and SLPI have been found in cervical mucus and in endocervical epithelium (Hein et al., 2002; Moriyama et al., 1999; Quayle et al., 1998; Svinarich et al., 1997; Valore et al., 1998). We therefore examined the expression of these and an expanded panel of AMPs reported to be expressed by human epithelial cells using real-time reverse transcription PCR (Kohlgraf et al., 2010; Wehkamp et al., 2007). Polarized A2EN cells expressed all AMP transcripts within 48 h of culture (Fig. 4a). Polarized A2EN cells expressed higher levels of the beta defensins (HBD 1–3) and SLPI, than the alpha defensins (HD5 and 6), or LL37 (Fig. 4a). Since TLR3 is highly expressed in A2EN cells, we stimulated cells with polyI:C, a potent TLR3 agonist, to determine the induced expression of AMPs in polarized A2EN cells. PolyI:C caused over a tenfold up-regulation of HBD2 and HBD3 transcripts in polarized A2EN cells ($p < 0.001$; Fig. 4b). HBD1, HD5, HD6, and SLPI transcripts were also significantly up-regulated upon polyI:C stimulation ($p < 0.05$; Fig. 4b). LL37 transcript levels from polyI:C-stimulated cells were not significantly different from those levels observed in unstimulated cells (Fig. 4b). Our results obtained with respect to HBD1, HBD2, HBD3, HD5, and SLPI are consistent with expression patterns seen in endocervical mucus and epithelial tissues (Hein et al., 2002; Moriyama et al., 1999; Quayle et al., 1998; Svinarich et al., 1997; Valore et al., 1998; Zapata et al., 2008). These results also report the novel expression of HD6 by endocervical epithelial cells, and polyI:C mediated induction of AMP mRNA expression.

3.5. Polarized A2EN cells secrete pro-inflammatory cytokines and chemokines when stimulated with polyI:C

The polarization of A2EN cells allows for dissection of the differential cytokine and chemokine responses from the apical and basolateral compartments of epithelial cells that cannot be examined using non-polarized cells grown on a traditional plastic surface. Therefore, we only utilized polarized A2EN cells for these studies. In order to determine differences in cytokine and chemokine secretion between apical and basolateral compartments, we stimulated polarized A2EN cells with polyI:C for 24 h and measured cytokines and chemokines in collected supernatants from both compartments.

Polarized A2EN cells constitutively expressed high levels of CXCL8, which has been previously reported in the female genital tract *in vitro* (Fahey et al., 2005; Fichorova and Anderson, 1999; Wira et al., 2011a,b) and *in vivo* (Kayisli et al., 2002; Lieberman et al., 2008; Simhan et al., 2003). Polarized A2EN cells also constitutively expressed high levels of IP10. Polarized A2EN cells showed a statistically significant induction of MIP1 β , RANTES, GCSF, IP10, IL6, CXCL8, and TNF α , in apical and basolateral supernatants when stimulated with polyI:C ($p < 0.05$; Fig. 5; Supp. Table 1); MIP1 β and RANTES were the most robustly secreted analytes observed. PolyI:C induced GM-CSF secretion in

basolateral, but not apical supernatants of polarized A2EN cells. IL1 α levels were only observed to be statistically significant in apical, but not basolateral supernatants in polyI:C-stimulated A2EN cells. These results indicate that polarized A2EN cells are capable of secreting pro-inflammatory cytokines and chemokines upon TLR 3 ligation, and that some of these cytokines and chemokines are differentially expressed from the apical and basolateral membrane compartments.

3.6. Female sex hormones regulate cytokine and chemokine secretion in polarized A2EN cells

A study in polarized endometrial epithelial cells found that LPS-induced secretion of pro-inflammatory cytokines and chemokines was inhibited upon estrogen exposure (Fahey et al., 2008). We therefore examined the hormonal regulation of cytokine and chemokine secretion by polarized A2EN cells. First, we examined the influence of estradiol or progesterone/estradiol on unstimulated polarized A2EN cells, but observed no alteration in constitutive cytokine or chemokine secretion in either apical or basolateral supernatants for any of the analytes that were measured (Fig. 6a–f and Supp. Table 1). Next, we examined the influence of hormones on the cytokine and chemokine secretion of polarized A2EN cells stimulated with polyI:C. Estradiol exposure had no effect on the polyI:C-induced secretion of any cytokines or chemokines measured from either the apical or basolateral supernatants from polarized A2EN cells (Fig. 6a–f and Supp. Table 1). Progesterone/estradiol exposure inhibited the polyI:C-induced secretion of MIP1 β , RANTES, IP10, TNF α , IL6, CXCL8, and GCSF, in both apical and basolateral supernatants from polarized A2EN cells (Fig. 6a–f and Supp. Table 1, $p < 0.05$). Progesterone/estradiol exposure only inhibited the polyI:C-induced secretion of IL1 α from the apical compartment, but not the basolateral compartment (Supp. Table 1, $p < 0.001$). Together these results suggest that the combination of hormones found in the secretory stage of the menstrual cycle modulate the ability of polarized A2EN cells to secrete pro-inflammatory cytokines and chemokines in response to TLR 3 ligation.

4. Discussion

We have described the development and characterization of a polarized epithelial cell culture model that produces many innate immune mediators including mucin, anti-microbial peptides, cytokines, and chemokines. This system uses early passages of the epithelial cell line A2EN that we previously derived from primary epithelial cells grown from human endocervix (Herbst-Kralovetz et al., 2008). Our studies indicate that polarized A2EN cells are hormonally responsive to physiological concentrations of estradiol and progesterone, yet non-polarized A2EN cells cultured on a plastic surface are less responsive. Furthermore, expression of MUC5B, a gel-forming mucin made by the endocervix, was either below the level of detection or not expressed in non-polarized A2EN cells whereas MUC5B was expressed in polarized A2EN cells. Polarized A2EN cells also differentially secreted certain cytokines such as IL1 α and GM-CSF from their apical and basolateral compartments. This is likely important *in vivo* because apically secreted proteins would be more likely to interact with apical surfaces of surrounding epithelial cells, while basally secreted proteins would most likely interact with stromal cells and underlying leukocytes. Taken together, these results suggest that A2EN cells architecturally structured in a polarized orientation have an expression profile of innate mediators that more closely resemble those expressed *in vivo* compared with non-polarized cells cultured on a plastic surface. Cells in a polarized orientation would also be useful in dissecting cell-to-cell communication pathways, both with respect to epithelial cell to epithelial cell and epithelial cell to stromal cell or leukocyte signaling. The latter cell types also have the potential to be included in an organotypic model using A2EN cells as the framework.

Interestingly, we observed a high constitutive expression of a number of key pro-inflammatory cytokines and chemokines in polarized A2EN cells. While we recognize that immortalization of epithelial cells by HPV oncogenes E6 and E7 might alter constitutive or induced cytokine and chemokine production, we have confirmed high constitutive levels of IP10 and CXCL8 secreted by HPV negative polarized primary endocervical epithelial cells (Supp. Fig. 3), which is supported by other *in vitro* studies that also reported high constitutive levels of cytokines in primary epithelial cells from mucosal sites (Fahey et al., 2005; Fichorova and Anderson, 1999; Herbst-Kralovetz et al., 2008; Wira et al., 2011a,b). Conversely, high constitutive cytokine levels were not observed in transformed cell lines such as HeLa cells (Rasmussen et al., 1997, unpublished observations). Furthermore, high levels of CXCL8 have been reported in cervical samples and other epithelial cell lines derived from the FRT, suggesting that the female genital tract might exhibit high baseline production of these cytokines (Fahey et al., 2005; Fichorova and Anderson, 1999; Kayisli et al., 2002; Lieberman et al., 2008; Simhan et al., 2003). Collectively, these findings suggest that the endocervix, and adjacent tissue sites in the FRT, constitutively produce baseline levels of some innate mediators *in vivo*. Whether there are regulatory mechanisms also present in the FRT that can modulate the expression or functional activity of innate immune mediators *in vivo* remains to be investigated.

Antimicrobial peptides have been demonstrated to be important for innate immune protection at mucosal surfaces including the FRT (reviewed in Beagley and Gockel, 2003). Although HBD1 is thought to be constitutively expressed (Ganz, 2003), we demonstrated that its expression was induced in response to TLR3 stimulation with polyI:C, similar to findings in uterine epithelial cells (Schaefer et al., 2005). While TLR3 activation is known to induce HBD2, HBD3, and SLPI in other cell types (Abrahams et al., 2006; Ganz, 2003), this is the first study to demonstrate modulation of these AMPs by polyI:C stimulation of TLR3 in polarized epithelial cells derived from endocervix, suggesting that there is a common mechanism of defensin regulation among different cell types. We also demonstrated induction of the alpha defensins, HD5 and HD6, in response to TLR3 stimulation in this study, for the first time. In contrast to defensins and SLPI, expression of LL37, or cathelicidin, was not altered in response to polyI:C. Expression of LL37 has been shown to be either induced or down-regulated in response to activation of many TLRs in other cell types (Bergman et al., 2005; Redfern et al., 2011; Rivas-Santiago et al., 2008). These results suggest that regulation of LL-37 expression might be dependent upon activation of specific pattern recognition receptors and/or the cell type by which it is expressed. AMPs have also been shown to both negatively and positively influence the invasion of sexually transmitted pathogens including *C. trachomatis*, *N. gonorrhoeae*, and HIV (Ding et al., 2009; Klotman et al., 2008; Porter et al., 2005). Our polarized A2EN epithelial cell model would be a useful model to delineate the regulation of these AMPs in response to TLR activation as well as pathogens *in vitro*.

The polarized A2EN epithelial cell model would be a useful tool for studying the innate immune responses and other epithelial cell interactions with sexually transmitted pathogens. *C. trachomatis* (serovars D-K), for instance, is an obligate intracellular bacteria that infects the epithelial cells of the upper FRT with the endocervix being the most common site of infection, and replication is generally limited to epithelial cells. Therefore, it has been hypothesized that the infected epithelial cells may be responsible for initiating and sustaining the immune response that is the primary mediator of chlamydial disease (Stephens, 2003). We have successfully infected polarized A2EN cells with *C. trachomatis* serovar D with an infection rate of >95% (data not shown). Hence, experiments can now be undertaken to characterize chlamydial infection and the epithelial cell immune response to this pathogen in a physiologically appropriate model. The polarized A2EN cell model could also be a useful tool to determine how other pathogens, such as *N. gonorrhoeae*, *M.*

genitalium, and HSV-2 establish and maintain infection, and how they modulate the subsequent innate immune responses in the endocervix.

Recent studies have indicated that the endocervix may be an important site in the transmission of HIV (Haase, 2010; Hladik and Hope, 2009; Li et al., 2009; Shacklett, 2009; Zhang et al., 1999). In the rhesus macaque model of SIV infection, lymphocytes positive for viral RNA were observed in the lamina propria of the endocervix following vaginal inoculation, suggesting a role of the endocervical epithelial cells as mediators in virus transmission to underlying susceptible leukocytes (Zhang et al., 1999). Recent studies reported similar findings whereby SIV crossed the epithelial cell barrier in the endocervix within hours of inoculation, and established small foci of infection (Haase, 2010; Li et al., 2009). Because polarized A2EN cells maintain elevated TEER measurements for up to three weeks in culture, they could be used to study HIV binding, transcytosis across the epithelial cell barrier, and recruitment and/or signaling to underlying leukocyte subsets that may contribute to virus transmission. This polarized system of endocervical epithelial cells could also be used for microbicide sensitivity studies and determining microbicide efficacy against a panel of pathogens that infect the endocervix.

In the upper female genital tract there must exist a delicate balance between an appropriate response to sexually transmitted pathogens and the provision of a tolerant environment that is thought to be necessary for conception, implantation, and the subsequent development of the allogeneic fetus. The mechanisms involved in tipping the balance toward an inflammatory versus a tolerant response in the FRT are extremely complex and most likely vary depending on the menstrual cycle status (Quayle, 2002; Wira et al., 2010, 2011a,b). Many clinical studies have found associations between stage of the menstrual cycle and susceptibility to disease (reviewed in Beagley and Gockel, 2003; Guinan et al., 1981; Ng et al., 1970; Sweet et al., 1986). The female sex hormones estradiol and progesterone regulate the stages of the menstrual cycle and modulate components of the innate and adaptive immune response that likely play a role in this delicate balance (Beagley and Gockel, 2003; Lea and Sandra, 2007; Nussey and Whitehead, 2001; Ochiel et al., 2008; Wira et al., 2010, 2011a,b). *In vivo* studies on *Chlamydia* spp. have demonstrated differences in susceptibility to infection of the endometrium depending on the stage of the menstrual cycle in swine (Guseva et al., 2003). *In vitro* experiments have demonstrated that estradiol enhances binding and infectivity of chlamydiae in endometrial epithelial cells whereas progesterone does not (Guseva et al., 2003; Maslow et al., 1988; Sweet et al., 1986). These studies have focused on hormonal modulation of chlamydial infection in the endometrium, but little is known about the influence of menstrual status or hormones on chlamydial infection in the endocervix. Since the endocervix lies between the microbe-rich lower reproductive tract and the relatively sterile upper tract, it likely plays a critical part in contributing to the immunological balance in the upper FRT by initiating immune responses that are influenced by female sex hormones and stage of the menstrual cycle. Since polarized A2EN cells are hormonally responsive, they could be used to examine the differences in infection, pathogenesis, and subsequent induction of endocervical epithelial cell immune responses by sexually transmitted pathogens, including HIV.

In the present study, we conducted preliminary experiments to determine how immune activation of A2EN cytokine and chemokine responses is regulated by female steroid hormones. To accomplish this, we established a hormonal milieu *in vitro* believed to resemble both the proliferative and the secretory phases of the menstrual cycle (Hombach-Klonisch et al., 2005). We then stimulated hormone-exposed polarized A2EN cells with polyI:C, a TLR3 agonist, as a model activator, with the rationale that TLR3 is highly expressed TLRs on endocervical epithelial cells, and polyI:C is one of the most potent TLR 3 agonists. We observed that estradiol alone had little to no effect on polyI:C-induced

secretion of pro-inflammatory cytokines and chemokines, but exposure to combined progesterone/estradiol significantly inhibited polyI:C induced pro-inflammatory cytokine and chemokine secretion. From these results it could be hypothesized that endocervix-derived epithelial cells under the influence of hormones of the secretory phase of the menstrual cycle might play a role in regulating inflammatory responses that might be detrimental to reproductive processes in the upper FRT. Interestingly, these results contrast with the results of a study undertaken in endometrial epithelial cells in which LPS- and polyI:C-induced secretion of pro-inflammatory cytokines and chemokines were inhibited upon estradiol exposure, while progesterone had no effect (Fahey et al., 2008). These contrasting results might be explained by the difference in tissue sites from which the cells were derived, further illustrating the importance of tissue-specific epithelial cell models. There may be different, yet specific, immunological regulatory mechanisms that exist in the endocervix compared with the endometrium. Hence, further studies are clearly needed using endocervical and endometrial epithelial cells from the same donor and with similar hormone priming protocols to fully understand the site-specific differences in the hormonal regulation of innate immune mediators in the FRT.

Finding that progesterone/estradiol exposure down-regulated pro-inflammatory responses was not entirely surprising, as numerous studies have demonstrated that progesterone has immuno-suppressive and immunomodulatory properties (Su et al., 2009; Hughes et al., 2008; Miyaura and Iwata, 2002). It has also been reported using animal modeling that progesterone increases susceptibility to some viral sexually transmitted pathogens (Kaushic et al., 2011; MacDonald et al., 2007). Progesterone has been shown to inhibit NF kappa B activation and up-regulate suppressor of cytokine signaling 1(SOCS1), which leads to inhibition of the secretion of pro-inflammatory cytokines (Su et al., 2009). Our results demonstrate that upon TLR 3 ligation, progesterone combined with estradiol also inhibits the secretion of the pro-inflammatory cytokines and chemokines, such as IL6, CXCL8, and TNF α in A2EN cells. Further studies need to be performed to determine which specific signaling events play a role in the hormone-mediated suppression of pro-inflammatory cytokines and chemokines in endocervical epithelial cells.

In conclusion, our studies indicate that A2EN cells polarize and provide a useful tool for examining the differences in the epithelial immune responses from both apical and basolateral compartments. Using this polarized endocervical cell model, we have demonstrated that endocervical epithelial cells likely play a role in the innate immune response of the FRT as they express numerous innate immune mediators including mucus, anti-microbial peptides, and proinflammatory cytokines and chemokines. We have also demonstrated that polarized A2EN cells are hormonally responsive, and that the female sex hormones estrogen and progesterone modulate the expression of proinflammatory cytokines and chemokines. In future studies, this model may be utilized in co-culture experiments to answer complex questions about sexually transmitted infections, including HIV, that involve cross-talk between endocervical epithelial cells and underlying stromal cells and leukocytes. Taken together, these polarized A2EN cells or similar models may serve as new tools to investigate the critical role of the endocervix in the immunological balance between protection and tolerance in the FRT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Abrahams VM, Schaefer TM, Fahey JV, Visintin I, Wright JA, Aldo PB, et al. Expression and secretion of antiviral factors by trophoblast cells following stimulation by the TLR-3 agonist, Poly(I:C). *Hum. Reprod.* 2006; 21:2432–2439. [PubMed: 16751646]
- Anderson DJ, Pudney J, Schust DJ. Caveats associated with the use of human cervical tissue for HIV and microbicide research. *AIDS.* 2010; 24:1–4. [PubMed: 19890206]
- Beagley KW, Gockel CM. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. *FEMS Immunol. Med. Microbiol.* 2003; 38:13–22. [PubMed: 12900050]
- Beatty WL. Trafficking from CD63-positive late endocytic multivesicular bodies is essential for intracellular development of *Chlamydia trachomatis*. *J. Cell Sci.* 2006; 119:350–359. [PubMed: 16410552]
- Belinson JL, Hu S, Niyazi M, Pretorius RG, Wang H, Wen C, et al. Prevalence of type-specific human papillomavirus in endocervical, upper and lower vaginal, perineal and vaginal self-collected specimens: implications for vaginal self-collection. *Int. J. Cancer.* 2010; 127:1151–1157. [PubMed: 20039323]
- Bergman P, Johansson L, Asp V, Plant L, Gudmundsson GH, Jonsson AB, et al. *Neisseria gonorrhoeae* downregulates expression of the human antimicrobial peptide LL-37. *Cell. Microbiol.* 2005; 7:1009–1017. [PubMed: 15953032]
- Brunham RC, Paavonen J, Stevens CE, Kiviat N, Kuo CC, Critchlow CW, et al. Mucopurulent cervicitis-the ignored counterpart in women of urethritis in men. *N Engl J Med.* 1984; 311:1–6. [PubMed: 6427611]
- Bryant DM, Mostov KE. From cells to organs: building polarized tissue. *Nat. Rev. Mol. Cell Biol.* 2008; 9:887–901. [PubMed: 18946477]
- Ding J, Chou YY, Chang TL. Defensins in viral infections. *J. Innate. Immun.* 2009; 1:413–420. [PubMed: 20375599]
- Espinosa M, Noe G, Troncoso C, Ho SB, Villalon M. Acidic pH and increasing [Ca(2+)] reduce the swelling of mucins in primary cultures of human cervical cells. *Hum. Reprod.* 2002; 17:1964–1972. [PubMed: 12151422]
- Fahey JV, Schaefer TM, Channon JY, Wira CR. Secretion of cytokines and chemokines by polarized human epithelial cells from the female reproductive tract. *Hum. Reprod.* 2005; 20:1439–1446. [PubMed: 15734755]
- Fahey JV, Wright JA, Shen L, Smith JM, Ghosh M, Rossoll RM, et al. Estradiol selectively regulates innate immune function by polarized human uterine epithelial cells in culture. *Mucosal Immunol.* 2008; 1:317–325. [PubMed: 19079193]
- Falk L, Fredlund H, Jensen JS. Signs and symptoms of urethritis and cervicitis among women with or without *Mycoplasma genitalium* or *Chlamydia trachomatis* infection. *Sex. Transm. Infect.* 2005; 81:73–78. [PubMed: 15681728]
- Fichorova RN, Anderson DJ. Differential expression of immuno-biological mediators by immortalized human cervical and vaginal epithelial cells. *Biol. Reprod.* 1999; 60:508–514. [PubMed: 9916021]
- Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol.* 2003; 3:710–720. [PubMed: 12949495]
- Gipson IK. Human endocervical mucins. *Ernst Schering Res.* 2005:219–244. Found. Workshop.

- Gipson IK, Spurr-Michaud S, Moccia R, Zhan Q, Toribara N, Ho SB, et al. MUC4 and MUC5B transcripts are the prevalent mucin messenger ribonucleic acids of the human endocervix. *Biol. Reprod.* 1999; 60:58–64. [PubMed: 9858486]
- Gipson IK, Moccia R, Spurr-Michaud S, Argueso P, Gargiulo AR, Hill JA 3rd, et al. The amount of MUC5B mucin in cervical mucus peaks at midcycle. *J. Clin. Endocrinol. Metab.* 2001; 86:594–600. [PubMed: 11158014]
- Guinan ME, MacCalman J, Kern ER, Overall JC Jr, Spruance SL. The course of untreated recurrent genital herpes simplex infection in 27 women. *N. Engl. J. Med.* 1981; 304:759–763. [PubMed: 6258073]
- Guseva NV, Dessus-Babus SC, Whittimore JD, Moore CG, Wyrick PB. Characterization of estrogen-responsive epithelial cell lines and their infectivity by genital *Chlamydia trachomatis*. *Microbes Infect.* 2005; 7:1469–1481. [PubMed: 16046168]
- Guseva NV, Knight ST, Whittimore JD, Wyrick PB. Primary cultures of female swine genital epithelial cells in vitro: a new approach for the study of hormonal modulation of *Chlamydia* infection. *Infect. Immun.* 2003; 71:4700–4710. [PubMed: 12874351]
- Haase AT. Targeting early infection to prevent HIV-1 mucosal transmission. *Nature.* 2010; 464:217–223. [PubMed: 20220840]
- Haase AT. Early events in sexual transmission of HIV and SIV and opportunities for interventions. *Annu. Rev. Med.* 2011; 62:127–139. [PubMed: 21054171]
- Halldorsson S, Asgrimsson V, Axelsson I, Gudmundsson GH, Steinars-dottir M, Baldursson O, et al. Differentiation potential of a basal epithelial cell line established from human bronchial explant. *In Vitro Cell. Dev. Biol. Anim.* 2007; 43:283–289. [PubMed: 17876679]
- Hein M, Valore EV, Helmig RB, Uldbjerg N, Ganz T. Antimicrobial factors in the cervical mucus plug. *Am. J. Obstet. Gynecol.* 2002; 187:137–144. [PubMed: 12114901]
- Hennings H, Michael D, Cheng C, Steinert P, Holbrook K, Yuspa SH. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell.* 1980; 19:245–254. [PubMed: 6153576]
- Herbst-Kralovetz MM, Quayle AJ, Ficarra M, Greene S, Rose 2nd WA, Chesson R, et al. Quantification and comparison of toll-like receptor expression and responsiveness in primary and immortalized human female lower genital tract epithelia. *Am. J. Reprod. Immunol.* 2008; 59:212–224. [PubMed: 18201283]
- Hickey DK, Patel MV, Fahey JV, Wira CR. Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. *J. Reprod. Immunol.* 2011; 88:185–194. [PubMed: 21353708]
- Hladik F, Hope TJ. HIV infection of the genital mucosa in women. *Curr. HIV/AIDS Rep.* 2009; 6:20–28. [PubMed: 19149993]
- Hombach-Klonisch S, Kehlen A, Fowler PA, Huppertz B, Jugert JF, Bischoff G, et al. Regulation of functional steroid receptors and ligand-induced responses in telomerase-immortalized human endometrial epithelial cells. *J. Mol. Endocrinol.* 2005; 34:517–534. [PubMed: 15821114]
- Hughes GC, Thomas S, Li C, Kaja MK, Clark EA. Cutting edge: progesterone regulates IFN- α production by plasmacytoid dendritic cells. *J Immunol.* 2008; 180:2029–2033. [PubMed: 18250406]
- Isaka K, Nishi H, Sagawa Y, Nakada T, Osakabe Y, Serizawa H, et al. Establishment of a new human cell line (EN) with TP53 mutation derived from endometrial carcinoma. *Cancer Genet. Cytogenet.* 2003; 141:20–25. [PubMed: 12581894]
- Joly S, Compton LM, Pujol C, Kurago ZB, Guthmiller JM. Loss of human beta-defensin 1, 2, and 3 expression in oral squamous cell carcinoma. *Oral Microbiol. Immunol.* 2009; 24:353–360. [PubMed: 19702947]
- Kaushic C, Roth KL, Anipindi V, Xiu F. Increased prevalence of sexually transmitted viral infections in women: the role of female sex hormones in regulating susceptibility and immune responses. *J. Reprod. Immunol.* 2011; 88:204–209. [PubMed: 21296427]
- Kayisli UA, Mahutte NG, Arici A. Uterine chemokines in reproductive physiology and pathology. *Am. J. Reprod. Immunol.* 2002; 47:213–221. [PubMed: 12069388]

- Klein TJ, Mlodzik M. Planar cell polarization: an emerging model points in the right direction. *Annu. Rev. Cell Dev. Biol.* 2005; 21:155–176. [PubMed: 16212491]
- Klotman ME, Rapista A, Teleshova N, Micsenyi A, Jarvis GA, Lu W, et al. *Neisseria gonorrhoeae*-induced human defensins 5 and 6 increase HIV infectivity: role in enhanced transmission. *J. Immunol.* 2008; 180:6176–6185. [PubMed: 18424739]
- Kohlgraf KG, Pingel LC, Dietrich DE, Brogden KA. Defensins as antiinflammatory compounds and mucosal adjuvants. *Future Microbiol.* 2010; 5:99–113. [PubMed: 20020832]
- Lea RG, Sandra O. Immunoendocrine aspects of endometrial function and implantation. *Reproduction.* 2007; 134:389–404. [PubMed: 17709558]
- Li Q, Estes JD, Schlievert PM, Duan L, Brosnahan AJ, Southern PJ, et al. Glycerol monolaurate prevents mucosal SIV transmission. *Nature.* 2009; 458:1034–1038. [PubMed: 19262509]
- Lieberman JA, Moscicki AB, Sumerel JL, Ma Y, Scott ME. Determination of cytokine protein levels in cervical mucus samples from young women by a multiplex immunoassay method and assessment of correlates. *Clin. Vaccine Immunol.* 2008; 15:49–54. [PubMed: 17978011]
- MacDonald EM, Savoy A, Gillgrass A, Fernandez S, Smieja M, Rosenthal KL, et al. Susceptibility of human female primary genital epithelial cells to herpes simplex virus, type-2 and the effect of TLR3 ligand and sex hormones on infection. *Biol. Reprod.* 2007; 77:1049–1059. [PubMed: 17881767]
- Maslow AS, Davis CH, Choong J, Wyrick PB. Estrogen enhances attachment of *Chlamydia trachomatis* to human endometrial epithelial cells in vitro. *Am. J. Obstet. Gynecol.* 1988; 159:1006–1014. [PubMed: 3177513]
- Miyaura H, Iwata M. Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids. *J. Immunol.* 2002; 168:1087–1094. [PubMed: 11801642]
- Mo B, Vendrov AE, Palomino WA, DuPont BR, Apparao KB, Lessey BA. ECC-1 cells: a well-differentiated steroid-responsive endometrial cell line with characteristics of luminal epithelium. *Biol. Reprod.* 2006; 75:387–394. [PubMed: 16707768]
- Moriyama A, Shimoya K, Ogata I, Kimura T, Nakamura T, Wada H, et al. Secretory leukocyte protease inhibitor (SLPI) concentrations in cervical mucus of women with normal menstrual cycle. *Mol. Hum. Reprod.* 1999; 5:656–661. [PubMed: 10381821]
- Ng AB, Reagan JW, Yen SS. Herpes genitalis; clinical and cytopathologic experience with 256 patients. *Obstet. Gynecol.* 1970; 36:645–651. [PubMed: 5506474]
- Nussey, SS.; Whitehead, SA. Ovarian control and the menstrual cycle. In: Nussey, SS.; Whitehead, SA., editors. *Endocrinology, An Integrated Approach*. Oxford, UK: BIOS Scientific Publishers Ltd; 2001. p. 255–261.
- Ochiel DO, Fahey JV, Ghosh M, Haddad SN, Wira CR. Innate immunity in the female reproductive tract: role of sex hormones in regulating uterine epithelial cell protection against pathogens. *Curr. Womens Health Rev.* 2008; 4:102–117. [PubMed: 19644567]
- Porter E, Yang H, Yavagal S, Preza GC, Murillo O, Lima H, et al. Distinct defensin profiles in *Neisseria gonorrhoeae* and *Chlamydia trachomatis* urethritis reveal novel epithelial cell-neutrophil interactions. *Infect. Immun.* 2005; 73:4823–4833. [PubMed: 16040996]
- Quayle AJ. The innate and early immune response to pathogen challenge in the female genital tract and the pivotal role of epithelial cells. *J. Reprod. Immunol.* 2002; 57:61–79. [PubMed: 12385834]
- Quayle AJ, Porter EM, Nussbaum AA, Wang YM, Brabec C, Yip KP, et al. Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *Am. J. Pathol.* 1998; 152:1247–1258. [PubMed: 9588893]
- Rasmussen SJ, Eckmann L, Quayle AJ, Shen L, Zhang YX, Anderson DJ, et al. 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.* 1997; 99:77–87. [PubMed: 9011579]
- Redfern RL, Reins RY, McDermott AM. Toll-like receptor activation modulates antimicrobial peptide expression by ocular surface cells. *Exp. Eye Res.* 2011; 92:209–220. [PubMed: 21195713]
- Rivas-Santiago B, Hernandez-Pando R, Carranza C, Juarez E, Con-treras JL, Aguilar-Leon D, et al. Expression of cathelicidin LL-37 during *Mycobacterium tuberculosis* infection in human alveolar

- macrophages, monocytes, neutrophils, and epithelial cells. *Infect. Immun.* 2008; 76:935–941. [PubMed: 18160480]
- Schaefer TM, Fahey JV, Wright JA, Wira CR. Innate immunity in the human female reproductive tract: antiviral response of uterine epithelial cells to the TLR3 agonist poly(I:C). *J. Immunol.* 2005; 174:992–1002. [PubMed: 15634923]
- Shacklett BL. Cell-mediated immunity to HIV in the female reproductive tract. *J. Reprod. Immunol.* 2009; 83:190–195. [PubMed: 19857902]
- Simhan HN, Caritis SN, Krohn MA, Martinez de Tejada B, Landers DV, Hillier SL. Decreased cervical proinflammatory cytokines permit subsequent upper genital tract infection during pregnancy. *Am. J. Obstet. Gynecol.* 2003; 189:560–567. [PubMed: 14520234]
- Soler C, Allibert P, Chardonnet Y, Cros P, Mandrand B, Thivolet J. Detection of human papillomavirus types 6, 11, 16 and 18 in mucosal and cutaneous lesions by the multiplex polymerase chain reaction. *J Virol Methods.* 1991; 35:143–157. [PubMed: 1667785]
- Stephens RS. The cellular paradigm of chlamydial pathogenesis. *Trends Microbiol.* 2003; 11:44–51. [PubMed: 12526854]
- Su L, Sun Y, Ma F, Lu P, Huang H, Zhou J. Progesterone inhibits Toll-like receptor 4-mediated innate immune response in macrophages by suppressing NF-kappaB activation and enhancing SOCS1 expression. *Immunol. Lett.* 2009; 125:151–155. [PubMed: 19607861]
- Svinarich DM, Wolf NA, Gomez R, Gonik B, Romero R. Detection of human defensin 5 in reproductive tissues. *Am. J. Obstet. Gynecol.* 1997; 176:470–475. [PubMed: 9065200]
- Sweet RL, Blankfort-Doyle M, Robbie MO, Schacter J. The occurrence of chlamydial and gonococcal salpingitis during the menstrual cycle. *JAMA.* 1986; 255:2062–2064. [PubMed: 3959289]
- Valore EV, Park CH, Quayle AJ, Wiles KR, McCray PB Jr, Ganz T. Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. *J. Clin. Invest.* 1998; 101:1633–1642. [PubMed: 9541493]
- Wang J, Frohlich KJ, Buckner L, Quayle AJ, Luo M, Feng X, et al. Altered protein secretion of *Chlamydia trachomatis* in persistently infected human endocervical epithelial cells. *Microbiology.* Jul 7.2011 Epub ahead of print.
- Wehkamp J, Schaubert J, Stange EF. Defensins and cathelicidins in gastrointestinal infections. *Curr. Opin. Gastroenterol.* 2007; 23:32–38. [PubMed: 17133082]
- Wira CR, Fahey JV, Ghosh M, Patel MV, Hickey DK, Ochiel DO. Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. *Am. J. Reprod. Immunol.* 2010; 63(6):544–565. [PubMed: 20367623]
- Wira CR, Ghosh M, Smith JM, Shen L, Connor RI, Sundstrom P, et al. Epithelial cell secretions from the human female reproductive tract inhibit sexually transmitted pathogens and *Candida albicans* but not *Lactobacillus*. *Mucosal Immunol.* 2011a; 4:335–342. [PubMed: 21048705]
- Wira CR, Patel MV, Ghosh M, Mukura L, Fahey JV. Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections. *Am. J. Reprod. Immunol.* 2011b; 65:196–211. [PubMed: 21294805]
- Wisniewski L, Jornot L, Dudez T, Pagano A, Rochat T, Lacroix JS, et al. Long-term cultures of polarized airway epithelial cells from patients with cystic fibrosis. *Am. J. Respir. Cell Mol. Biol.* 2006; 34:39–48. [PubMed: 16179582]
- Wyrick, P. Polarized epithelial cell culture for *Chlamydia trachomatis*. In: Wyrick, P.; Bavoi, P., editors. *Chlamydia Genomics and Pathogenesis*. Norfolk, UK: Horizon Bioscience; 2006. p. 323–338.
- Yuan JS, Reed A, Chen F, Stewart CN Jr. Statistical analysis of real-time PCR data. *BMC Bioinformatics.* 2006; 7:85. [PubMed: 16504059]
- Zapata W, Rodriguez B, Weber J, Estrada H, Quinones-Mateu ME, Zimmermann PA, et al. Increased levels of human beta-defensins mRNA in sexually HIV-1 exposed but uninfected individuals. *Curr. HIV Res.* 2008; 6:531–538. [PubMed: 18991618]

- Zhang Z, Schuler T, Zupancic M, Wietgreffe S, Staskus KA, Reimann KA, et al. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science*. 1999; 286:1353–1357. [PubMed: 10558989]
- Zhu Y, Maric J, Nilsson M, Brannstrom M, Janson PO, Sundfeldt K. Formation and barrier function of tight junctions in human ovarian surface epithelium. *Biol. Reprod.* 2004; 71:53–59. [PubMed: 14973266]

Fig. 1.

A2EN cells polarize and form tight junctions. (a) Hematoxylin and eosin staining of paraffin embedded A2EN cells on a transwell membrane. (b) TEM image of A2EN cells in a monolayer on transwell membranes. (c) TEM high magnification image of junctional complexes in polarized A2EN cells. Scale bars in TEM images represent 1 μm . (d) Fluorescent image (1000 $\times$ magnification) of claudin-4 (green) protein, a protein in the tight junction complex in polarized A2EN cells after five days under differentiation conditions. Scale bar represents 5 μm . (e) TEER values of A2EN cells from days 1 to 12 under differentiation conditions indicate tight junction formation. Data points on the graph

represent the means $\pm$ the standard deviation (SD) of three independent experiments each performed in triplicate.

Fig. 2.

Polarized A2EN cells generate gel-forming mucin. (a) RT-PCR for MUC5B in polarized A2EN cells, non-polarized A2EN cells cultured on plastic, and HeLa 229 cells. Endocervical tissue (endo tissue) was used as a positive control. Water was used as a blank control. (b) Deconvolution fluorescent image (400× magnification) of MUC5B (green in the merged image) protein on the apical surface of polarized A2EN cells. Cell nuclei were stained with DAPI (grey). Scale bars represent 5 μm. Figures are representative of at least three independent experiments.

Fig. 3.

Polarized A2EN cells express hormone receptors and respond to female sex hormones. (a) Polarized A2EN cells were exposed to either PBS or tamoxifen for 2 h at 37 °C. Cells were then exposed to 10^{-8} M estradiol for 24 h at 37 °C. ER α translocates to the nucleus in polarized A2EN cells exposed to estradiol in the absence of tamoxifen. Tamoxifen exposure abrogated ER α translocation to the nucleus in a dose-dependent manner. Labels indicate molar concentrations of tamoxifen. ER β immunostaining revealed similar results (data not shown). Scale bars represent 5 μ m. (b) Quantitative RT-PCR of PGR with female sex hormone exposure. Estradiol exposure resulted in an increase of PGR mRNA. Progesterone/estradiol exposure resulted in a decrease in PGR expression. Values shown are the mean

PGR levels $\pm$ SD from triplicate samples. The figure shown is a representative graph from three independent experiments. Statistical comparisons (ANOVA) between control and hormone-exposed samples were performed (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fig. 4.

Quantification of constitutive and induced anti-microbial peptide transcripts in polarized A2EN cells. (a) Expression of HBD1-3, HD5 and 6, SLPI, and LL37 was measured 48 h after medium was changed. AMP expression levels were normalized to 18srRNA expression, and then multiplied by 10^7 for ease of visualization. (b) Polarized A2EN cells were stimulated with 100 µg/ml polyI: C for 24 h. Graph represents the fold induction of polyI:C stimulated AMP expression in polarized A2EN cells. Induced levels of AMPs were normalized to AMP expression levels in the unstimulated control. All AMP values shown are the mean levels $\pm$ SD from triplicate samples from at least three independent experiments for each AMP. Statistical comparisons (ANOVA) between polyI:C stimulated and unstimulated cultures were performed (* p < 0.05, ** p < 0.01, *** p < 0.001).

Fig. 5.

Cytokine and chemokine secretion by polarized A2EN cells in response to the TLR 3 agonist, polyI:C. Polarized A2EN cells were stimulated with 100 µg/mL polyI:C for 24 h. Apical and basolateral supernatants were analyzed by cytometric bead array to quantify 10 different analytes. White bars represent control concentrations. Black bars represent polyI:C stimulated concentrations. Values shown are the mean cytokine and chemokine levels $\pm$ SD from triplicate samples from at least three independent experiments. Statistical comparisons (ANOVA) between polyI:C stimulated and unstimulated cultures were performed (* $p < 0.05$ or ** $p < 0.001$).

Fig. 6. Cytokine and chemokine regulation by female sex hormones in polarized A2EN cells. Polarized A2EN cells were exposed to 10^{-8} M estradiol or 10^{-7} M progesterone/ 10^{-9} M estradiol for 24 h. Cells were then stimulated with 100 μ g/mL polyI: C for 24 h. (a) IL6, (b) CXCL8, (c) TNF α , (d) IP10, (e) MIP1 β , and (f) RANTES are shown. Cytokine and chemokine values shown are the mean levels $\pm$ SD from triplicate samples from three independent experiments. Statistical comparisons (ANOVA) of all experimental conditions were performed (* p < 0.05, ** p < 0.001).