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Differential induction of innate anti-viral responses by TLR ligands against Herpes simplex virus, type 2, infection in primary genital epithelium of women

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ABSTRACT

Genital epithelial cells (GECs) are the first line of mucosal defense against sexually transmitted infections. We exploited the ability of GECs to mount innate immune responses, by using TLR ligands to induce anti-viral activity against Herpes simplex virus, type 2 (HSV-2). Primary cultures of GECs were grown to confluent, polarized monolayers and found to express different levels of mRNA for TLR1–10. Innate anti-viral responses against HSV-2 infection were determined following treatment with eight different TLR ligands. HSV-2 replication was significantly inhibited following treatment with ligands for TLR3, 5 and 9, while lipo-polysaccharide (LPS), a TLR4 ligand, failed to provide any protection. Biologically active interferon-beta and nitric oxide production by GECs correlated with anti-viral activity. Following treatment with TLR3 ligand Poly I:C, inflammatory cytokines were upregulated. Poly I:C treatment led to activation of downstream transcription factors including interferon regulatory factor-3 (IRF-3) and NFκB. Anti-viral responses induced by TLR ligands in GECs may provide a unique alternative to topical microbicides by enhancing body's own mucosal innate defense mechanisms against sexually transmitted viruses.

1. Introduction

Herpes simplex virus (HSV) infections are among most common sexually transmitted genital infections. HSV-2 has an estimated sero-prevalence of 20% in North American adult population and 30–80% among women in developing countries and sub-Saharan Africa (Paz-Bailey et al., 2007; Smith and Robinson, 2002). HSV-2 is transmitted primarily by symptomatic lesions and asymptomatic shedding following genital infection. Primary infection in epithelium is followed rapidly by entry of the virus into the central nervous system by entering sensory neurons and establishment of latent infection and periodic re-activation (Corey et al., 1983). Genital herpes is a life-long infection that can cause substantial morbidity to those infected and have serious consequences, including neonatal herpes and increased risk for HIV acquisition and transmission (Corey and Wald, 1999). Current treatment options are limited to several anti-viral therapies that control re-activation of the virus. However, these treatments do not eliminate latent virus (Corey et al., 2007; Paz-Bailey et al., 2007; Simmons, 2002). Currently no vaccines are available to prevent genital HSV-2 infections (Haddow and Mindel, 2006; Koelle, 2006).

More recently there has been considerable interest in development of topical microbicides that can prevent transmission and infection of sexually transmitted infection (Balzarini and Van Damme, 2007; van de Wijgert and Shattock, 2007). Traditional microbicides act by killing the microorganism. However, new promising approaches to microbicides is expanding this definition to include classes of compounds that disrupt cell membrane of microorganisms, block receptor-mediated entry of pathogens, inhibit or intercept replication pathways or enhance innate antimicrobial pathways to make host cells resistant (Balzarini and Van Damme, 2007; Corey et al., 2007) (McGowan, 2006). The use of TLR ligands to activate anti-viral responses is a promising strategy (Herbst-Kralovetz and Pyles, 2006a). A number of studies have shown the efficacy of intra-vaginal delivery of CpG oligonucleotides, recognized by TLR9, to protect against genital herpes infection in mice (Ashkar et al., 2003; Harandi et al., 2003; Pyles et al., 2002; Sajic et al., 2003). Similarly, polyinosine–polycytosine

Abbreviations: GEC, genital epithelial cells; TLR, toll-like receptor; Poly I:C, polyionisinic–polycytidylic acid; CpG, oligonucleotides containing CpG motifs; LPS, lipopolysaccharide; NO, nitric oxide; IFN, interferon; VSV, vascular stomatitis virus; HSV-2, Herpes simplex virus, type 2; IRF-3, interferon regulatory factor-3; TER, transepithelial electrical resistance.

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acid (Poly I:C), a synthetic double-stranded RNA that binds to TLR3, leads to protection against herpes in mouse models (Ashkar et al., 2004; Herbst-Kralovetz and Pyles, 2006b). In human preclinical and clinical studies, imiquimod, a TLR7/8 ligand, has been tested for treatment of genital herpes with varied success (Abbo et al., 2007; Yudin and Kaul, 2008).

Human genital epithelial cells are the first line of defense against sexually transmitted pathogens (Wira et al., 2005). These cells play a key role in innate immunity. They respond to "danger signals" such as pathogens by producing an array of innate factors including cytokines and chemokines that initiate immune responses (Wira et al., 2005). Studies have shown that both upper and lower female reproductive tract epithelial cells express toll-like receptors (Andersen et al., 2006; Fichorova et al., 2002; Herbst-Kralovetz et al., 2008; Schaefer et al., 2005). Treatment with TLR3 and TLR9 agonists resulted in upregulation of cytokines and anti-viral factors (Andersen et al., 2006; Schaefer et al., 2005). A recent study examined the effect of TLR ligands on inhibition of human cytomegalovirus replication in human foreskin fibroblasts and ectocervical tissues (Fox-Canale et al., 2007).

In the present study, we examined the anti-viral responses induced in GECs following treatment with TLR ligands, in an ex vivo primary culture model. The ability of eight different TLR ligands to induce anti-viral responses in GECs against HSV-2 infection was determined. We found that treatment with different TLR ligands induced differential anti-viral responses and reduced HSV-2 replication in genital epithelial cells. GEC cultures treated with TLR3 ligand (Poly I:C), TLR9 ligand (CpG A) and TLR5 ligand (flagellin) showed greatest reduction in HSV-2 replication (80–100%) compared to untreated controls. Treatment with TLR4 ligand (LPS) failed to show any decrease in viral replication. Poly I:C treatment induced maximum production of both interferon-β and NO, post-treatment. Inflammatory cytokines, IL-1β, IL-6 and TNF-α, were significantly induced in GECs 24–48 h following Poly I:C treatment, but not with other TLR ligands. Further determination of the pathways of cytokine induction showed activation and nuclear translocation of both NF-κB and IRF-3 within 2 h of treatment with Poly I:C. This study provides direct evidence that innate activation of GECs by TLR ligands can protect against sexually transmitted viruses. This information will be useful in development of microbicides and vaccine adjuvants against sexually transmitted infections.

2. Materials and methods

2.1. Source of tissues

Reproductive tract tissues were obtained from women aged 30–59 years (mean age 42.9 ± 7.2) undergoing hysterectomy for benign gynecological reasons at Hamilton Health Sciences Hospital. Informed consent was obtained with the approval of Hamilton Health Sciences Research Ethics Board. The most common reasons for surgery were uterine fibroids and heavy bleeding.

2.2. Human primary genital epithelial cell isolation and cultures

Detailed protocol for isolation and culture of genital epithelial cells has been described previously (Macdonald et al., 2007). Briefly, endometrial and cervical tissues were obtained from women undergoing hysterectomy and minced into small pieces and digested in an enzyme mixture for 1 h at 37 °C. Epithelial cells (EC's) were isolated by a series of separations through nylon mesh filters of different pore sizes. EC's were finally grown onto Matrigel™ (Becton, Dickinson and Company) coated, 0.4-μm pore-size polycarbonate membrane tissue culture inserts (BD Falcon, Mississauga, Canada) with primary tissue culture medium (DMEM/F12; Invitrogen, Canada) supplemented with 10 μM HEPES (Invitrogen, Canada), 2 μM L-glutamine (Invitrogen, Canada), 100 units/ml penicillin/streptomycin (Sigma–Aldrich, Oakville, Canada), 2.5% Nu Serum culture supplement (Becton, Dickinson and company, Franklin Lakes, USA), and 2.5% Hyclone defined fetal bovine serum (Hyclone, Logan, USA). Polarized monolayers were formed within 5–7 days. The confluency of EC cultures was monitored by trans-epithelial resistance (TER) across monolayers measured by a Volt ohm meter (EVOM; World Precision Instruments, Sarasota, FL, USA). Epithelial monolayers showing TER values higher than 1000 Ω/cm were considered completely confluent and used for further experiments. The methodology used for monitoring the purity of the epithelial monolayers has been described before (Macdonald et al., 2007). The purity of GEC monolayers was between 95% and 98%. There was no trace of any hematopoietic cells in the confluent monolayers.

2.3. Semi-quantitative RT PCR

The expression of TLR1–10 in untreated GEC monolayers was assessed by semi-quantitative RT PCR. Total RNA was extracted from GECs with TRIzol reagent (Invitrogen, Burlington, Canada) according to manufacturer's instructions and mRNA was then converted to complementary DNA (cDNA) by reverse transcription using Superscript II kit (Invitrogen, Burlington, Canada). Oligonucleotide primer sequences (Mobix, McMaster University) are listed in Table 1. PCR was performed by an initial denaturation at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min. Final extension was at 72 °C for 5 min. PCR products were then electrophoresed on 2% agarose gel in 1× TAE buffer. The gel was stained with 1 μg/ml ethidium bromide at room temperature for 30 min and de-stained in water and photographed with UV transilluminator (Gel Doc 2000, Bio-Rad, Mississauga, ON). Densities of DNA bands were quantified to signal volumes using ImageQuant 5.0. Expressed amount of PCR product volume (signal unit/bp), was calculated and normalized

Table 1
Semi-quantitative PCR primers for detection of human TLRs.

Target genes	Primer	Sequence (5′–3′)
TLR1	Forward	CTT ATA AGT GTG ACT ACC CGG
	Reverse	CCA CAA TGC TCT TGC CAG G
TLR2	Forward	GTT AAC AAT CCG GAG GCT GC
	Reverse	TTG GGA ATG CAG CCT GTT AC
TLR3	Forward	CCC TTG CCT CAC TCC CC
	Reverse	CCT CTC CAT TCC TGG CCT
TLR4	Forward	CTG GAC CTC TCT CAG TGT C
	Reverse	GGC AGA GCT GAA ATG GAG G
TLR5	Forward	TGG GGG AAC TTT ACA GTT CG
	Reverse	CTG GGA TTC TCT GAA GGG G
TLR6	Forward	GGG TTG AGA GTA TAG TGG TG
	Reverse	GTA GAT GCA GAG GGA GGT C
TLR7	Forward	CCT CAG CCA CAA CCA ACT G
	Reverse	TTG TGT GCT CCT GGC CCC
TLR8	Forward	AAA CTT GAG CCA CAA CAA CAT TT
	Reverse	ATC TCC AAT GTC ACA GGT GC
TLR9	Forward	GGA GGG GAG AAG GTC TGG
	Reverse	CAA AGG GCT GGC TGT TGT AG
TLR10	Forward	AAA ACT CTA AAT GCG GGA AGA AA
	Reverse	GAA ATA AAT GCG TGG AAT CGG A
h β-Actin	Forward	CTG GAG AAG AGC TAC GAG C
	Reverse	GTG GAC AGC GAG GCC AG

to house keeping gene, β-actin and plotted as % relative expression.

2.4. Treatments of GECs with TLR ligands

To determine the effect of TLR ligands, GECs from endometrial and cervical tissues were grown to confluence. The average tissue yield was sufficient to grow approximately 8–18 culture inserts at a seeding density of $(1–2) \times 10^5$ cells per insert. Primary GEC cultures were incubated for 24 h with various TLR ligands: TLR2 ligand: peptidoglycan (PG; 10 μg/ml; Fluka, Sigma–Aldrich, Oakville, Canada); TLR2 ligand: lipoteichoic acid (LTA; 10 μg/ml); TLR3 ligand: polyinosinic–polycytidylic acid (Poly I:C; 25 μg/ml; Sigma–Aldrich, Oakville, Canada); TLR4 ligand: lipopolysaccharide (LPS; 100 ng/ml; Sigma–Aldrich, Oakville, Canada); TLR5 ligand: recombinant Flagellin (10 μg/ml; prepared as previously described, (Lauzon et al., 2006). TLR9 ligand: unmethylated CpG containing oligos or control ODNs (10 μg/ml; Table 2) was added to GEC monolayers in primary medium. The specific concentrations of TLR ligands used in these studies were based on previous in vivo and in vitro studies from our group and others (Ashkar et al., 2003, 2004; Gill et al., 2006; Macdonald et al., 2007) (Andersen et al., 2006; Schaefer et al., 2005). Apical and basolateral supernatants were collected at various time points post-treatment and biologically active interferon, cytokines and nitric oxide were measured.

2.5. HSV-2 infection and titration

Following 24 h treatment with TLR ligands, the supernatants of GEC culture were collected. The monolayers were washed and infected with HSV-2 strain 333 (10^4 PFU/well) which corresponds to an MOI in the range of 0.1–0.2. After 2 h of infection, monolayers were washed three times with PBS and fresh primary culture medium was added. Infected monolayers were incubated for further 24 h, after which supernatants were collected for viral titration. HSV-2 viral shedding in the supernatants was titrated on vero cells by plaque assay as described previously (Macdonald et al., 2007). At least three replicates were run for each experimental treatment and were compared with medium-treated monolayers, as controls from the same tissue. The inter-tissue and intra-tissue variability and reproducibility has been described previously (Macdonald et al., 2007).

2.6. Measurement of nitric oxide production

Following treatment with TLR ligands, concentration of the oxidized metabolites of NO, nitrite (NO_2^-) in the supernatants of GEC cultures was assessed by Griess reaction as described previously (Bussiere et al., 2005). Briefly, supernatants were added to a 96-well plate along with known standards made with sodium nitrite solution. Griess reagent (Sigma, USA) was prepared according to the manufacturer's instructions and added to standards and samples. Plates were read at 550 nm. The concentration of NO in samples was calculated based on the standard curve.

2.7. VSV plaque reduction assay and anti-viral assay

To assess the presence of biologically active interferon in GEC supernatants, a VSV plaque reduction assay was used. This method is based on assaying the ability of VSV-GFP, a lytic but IFN-sensitive virus that expresses GFP under the control of a virus promoter (provided by Dr. B. Lichty, McMaster University), to replicate within cultures. This assay has been used previously to assess the presence of biologically active IFN (Paladino et al., 2006). Briefly, the supernatants were collected from primary epithelial cells after 24 h of ligand treatments and diluted with α-MEM medium, and added to plates with vero cells grown to 80% confluency. The vero culture plates were then incubated at 37 °C. 24 h later samples were removed from each well & the vero cells were challenged with vesicular stomatitis virus expressing GFP (VSV-GFP) for an hour. After an hour the unattached VSV-GFP was removed and vero cells were overlayed with 2% methylcellulose/2× F11 MEM medium (1:1 ratio) & incubated for 48 h. Levels of GFP fluorescence were visualized and quantified using a Typhoon scanner (Amersham Bioscience, GE Healthcare Bio-Sciences Corp., Piscataway, USA). Fluorescence was inversely proportional to the interferon activity. The fluorescence reading of treated cultures was compared with control cultures (no interferon) and presented as relative fluorescence.

2.8. Interferon-β neutralization

GECs were grown to confluence and treated with Poly I:C alone (25 μg/ml), with Poly I:C + IFN-β neutralizing antiserum (polyclonal rabbit anti-human IFN-β, Chemicon Labs, USA) (2×10^4 neutralization units/ml) or with Poly I:C + normal rabbit serum (diluted 1:500 to obtain same concentration as IFN antibody) for 24 h. Poly I:C, Poly I:C + Ab and (Poly I:C) untreated control cultures were then infected with HSV-2, 333 (10^4 PFU/well) for 2 h. Viral inoculum was washed off, fresh media was added and supernatants were collected 24 h post-infection. HSV-2 replication was measured in the collected supernatants by viral plaque assays.

2.9. Cytokine analysis

Apical and basolateral supernatants were analyzed for multiple cytokines using the Luminex multianalyte technology (Luminex Corporation, Austin, TX, USA) as described before (Fernandez et al., 2007). Multiplex bead-based sandwich immunoassay kits (Upstate Biotech, Millipore, MA, USA) were used to measure levels of IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α, as per the manufacturer's instructions. Parallel ELISA kit (R&D, Minneapolis, MN, USA) for TNF-α was performed to confirm Luminex results. Cut off limit for all the cytokines was 5 pg/ml and levels detected at or below this limit were considered and reported as undetectable.

2.10. Immunofluorescence microscopy

Confluent primary GEC cultures were treated with Poly I:C for 2, 4 and 8 h. Positive controls for IRF-3 staining consisted of Sendai virus infected cultures. Confluent GEC cultures were inoculated with Sendai virus at final concentration of 1 hemagglutinating unit/10^3 cells for 1 h. Excess virus was washed off, fresh media was added and infection was allowed to continue for 8 h. Following Poly I:C treatment or Sendai virus infection, epithelial monolayers were fixed with 10% buffered formalin and permeabilized with 0.1% Triton X-100. Primary antibody was rabbit polyclonal anti-human NFκB p65 (Santa Cruz Biotechnology Inc., Santa Cruz, USA) and polyclonal rabbit anti-IRF-3 antibody 15-02 (kindly provided by Michael David, University of California, San Diego, Department of Biology, California). Secondary antibody, Alexa Fluor 488 conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) was added to monolayers for 1 h at room temperature. Propidium iodide was used for nuclear counter-staining. Following extensive washing, filters were excised from the polystyrene filter supports, and mounted on glass slides in Gel/Mount anti-fade reagent (Biomeda Corp., Foster City, CA). Images were acquired using an inverted laser-scanning confocal microscope (LSM 510, Zeiss, Oberkochen, Germany).

Table 2
CpG oligos and their sequences.

Type of CpG	Sequence 5′–3′	Backbone
Type A 2216-CpG	G*G*G–G–G–A–C–G–A–T–C–G–T–C–G*G*G*G*G*	Mixed backbone
Type A control 2243-ODN	G*G*G–G–G–A–G–C–A–T–G–C–T–G–G*G*G*G*G*	Mixed backbone
Type B 2006-CpG	T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*T*G*T*C*G*T*T*	Phosphorothioate backbone
Type B control 2006-ODN	T*G*C*T*G*C* T*T*T*T*G*T*G*C* T*T*T*T*G*T*G*C*T*T*	Phosphorothioate backbone
Type C 2395-CpG	T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G*	Phosphorothioate backbone
Type C control-ODN	T*G*C*A*G*G*A*C*T *T*C*T*C*T*C*A*G*G*T*T*	Phosphorothioate backbone

(*) Phosphorothioate linkage; (–) phosphodiester linkage.

2.11. Statistical analysis

GraphPad Prism version 4 (GraphPad Software, San Diego, CA) was used to compare three or more means by two-way analysis of variance (ANOVA). When an overall statistically significant difference was seen, post-tests were performed to compare pairs of treatments, using the Bonferroni method to adjust the *p*-value for multiple comparisons. For comparison of two means, unpaired or paired *t*-tests were performed. An alpha value of 0.05 was set for statistical significance. *p*-Values for each analysis are indicated in figure legends.

3. Results

3.1. Expression of TLRs by primary genital epithelial cells (GECs)

The expression of TLRs in genital tract has previously been described both in vivo and in vitro (Andersen et al., 2006; Fichorova et al., 2002; Herbst-Kralovetz et al., 2008; Schaefer et al., 2005). However, depending on differences in the source of tissues, culture conditions and purity of epithelial monolayers there are distinct differences in TLR levels reported in these studies. We therefore decided to first determine the constitutive expression of TLRs in the GECs, under our culture conditions. The culture conditions and purity of GEC monolayers used in our studies have been previously reported (Macdonald et al., 2007). Primary endometrial EC cultures were >98% pure and primary cervical ECs were >95% pure (Macdonald et al., 2007). Epithelial monolayers were grown from endometrial and cervical tissues for 5–7 days until they reached confluence. RNA was extracted from confluent epithelial cultures and analyzed for the expression of TLRs 1–10 by semi-quantitative RT-PCR. As compared to cervical epithelial cells, overall constitutive expression of TLRs was lower in endometrial epithelial cells (Fig. 1). TLR4, 7, 8 and 10 were expressed at minimal levels in endometrial cells. In comparison, TLR1, 2, 3, 5, 6 and 9 were expressed at significantly higher levels ($p < 0.001$). In cervical epithelial cultures constitutive expression of TLR2, 5, 8, 9 and 10 was minimal while TLR1, 3, 4, 6 and 7 were present at significantly higher levels ($p < 0.001$).

3.2. Reduction in HSV-2 replication following TLR ligand treatment of GECs

We next determined whether treatment with TLR ligands induced anti-viral effects in GECs and resulted in decreased HSV-2 replication. Eight different TLR ligands were examined for their anti-viral effect against HSV-2 replication (Fig. 2A). Confluent endometrial EC monolayers were treated with individual TLR ligand for 24 h and then infected with HSV-2. Two ligands were used for TLR2, lipoteichoic acid (LTA) and peptidoglycan (PG). HSV-2 replication was reduced by 56% and 50% when epithelial monolayers were treated with the PG and LTA, respectively. TLR3 ligand, synthetic double-stranded RNA, polyinosine–polycytidylic acid (Poly I:C) was found to be very effective in controlling HSV-2 replication; viral titers were reduced by >99.5%. LPS, a TLR4 ligand, failed to reduce HSV-2 replication and GECs were found unresponsive to LPS stimulation. TLR5 ligand, flagellin protein, reduced HSV-2 replication by 80%.

Three different CpG ODNs were examined alongside their non-CpG ODN controls as TLR9 ligands. As there is very little information available about interaction between Type A, B and C CpG ODNs

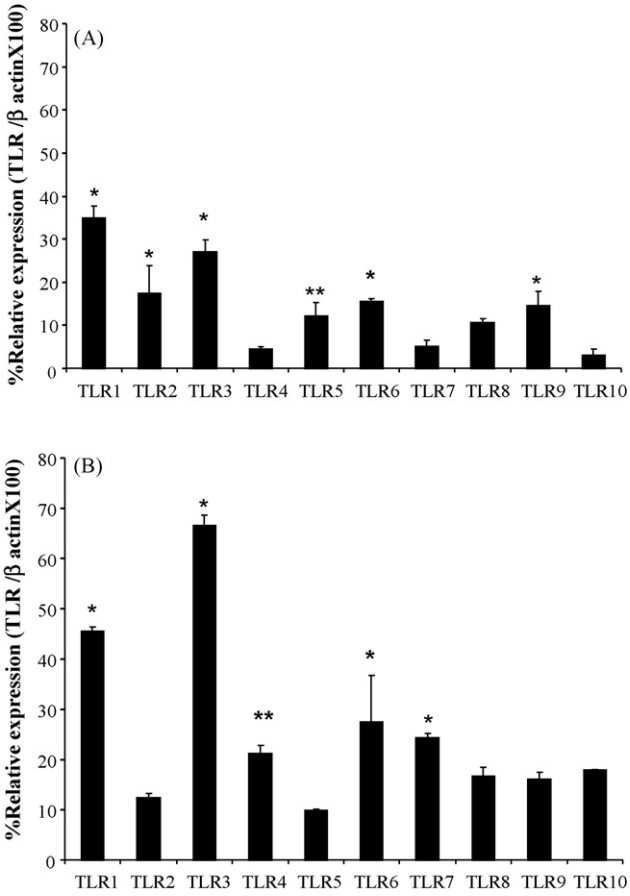

Fig. 1. Expression of TLRs in endometrial and cervical epithelial cells. Normal expression of TLR1–10 measured by semi-quantitative PCR in primary cultures of human endometrial (A) and cervical epithelial cells (B). Primary GEC cultures were grown to confluence, total RNA was extracted and TLR1–10 were examined from three replicate wells from each tissue sample. Expressed amount of PCR product volume (signal unit/bp), was calculated and normalized to house keeping gene, β-actin and plotted as % relative expression. In endometrial epithelial cells TLR4, 7, 8 and 10 were expressed in low amounts. TLR1, 2, 3, 6 and 9 was expressed significantly higher (*$p < 0.001$) than TLR10. TLR5 was also significantly higher than TLR10 (**$p < 0.01$) in endometrial cells. In cervical cells when compared to TLR2, TLR1, 3, 6 and 7 were expressed significantly higher (*$p < 0.001$). TLR4 was also significantly higher (**$p < 0.01$). Data shown is from three different GEC cultures of endometrium and cervix. The C.V. values for TLR mRNA expression in endometrial cultures was <10% (TLR1–8) and 17.5% and 18.7% for TLR9 and 10, respectively. C.V. values for cervical cultures were ≤10% (TLR1–10).

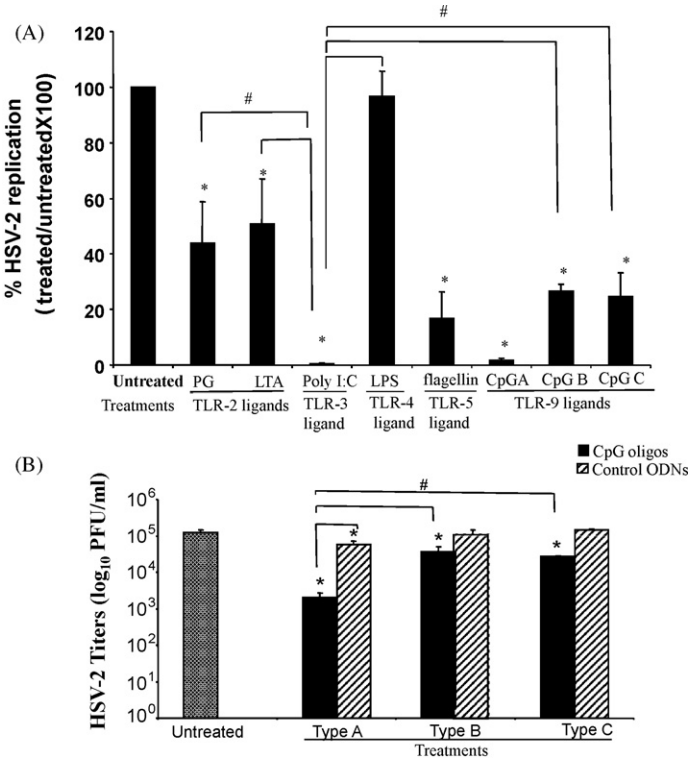

Fig. 2. Protection against HSV-2 infection following TLR ligand. (A) Endometrial epithelial monolayers were treated with different TLR ligands for 24 h. Ligand-treated and untreated (medium treated) control monolayers were infected with HSV-2, 333 (10^4 PFU/well) at an approximate MOI of 0.1. The reduction in viral titers in TLR ligand treated, infected monlayers was compared with untreated, infected controls. Reduction in viral counts following different ligand treatments was plotted as percent of untreated samples, in viral titers to allow comparison between different patient cultures. Each treatment group was run in triplicate wells for GEC culture and data shown is from four to seven different GEC cultures. Viral counts were significantly reduced following treatment with all TLR ligands compared to untreated controls with the exception of LPS ($^*p < 0.001$). Poly I:C provided significantly higher protection when compared to peptidoglycan (PG), lipoteichoic acid (LTA) and lipopolysaccharide (LPS), CpG B and CpG C treatments ($^\#p < 0.01$). (B) GEC monolayers were treated with Type A, B and C CpG oligonucleotides at a concentration of 10 μg/ml with the appropriate control ODNs at the same concentration for 24 h. Triplicate wells were run for each different treatment. Supernatants containing CpGs was removed and monolayers were infected with HSV-2, 333 (10^4 PFU/well), approximate MOI of 0.1. HSV-2 viral shedding was assessed by plaque assay. $^*p < 0.001$ compared to untreated control. $^\#p < 0.01$ compared to Type A CpG. Black bars indicates CpG oligos and lined bars indicates control ODNs for each CpG type. Data shown is representative of one of three separate experiments with similar results.

with GECs, we compared all three. Based on differences in their sequences, length, number of CpG motifs, chemical modifications and tertiary structures, the three types of CpGs appear to have different immunostimulatory capacity. The B-class ODNs are very potent Th1 adjuvants, stimulate strong B-cells and NK cell activation, plasmacytoid DC (pDC) maturation but induce only low amounts of IFN-α/-β secretion (Gursel et al., 2002; Hartmann and Krieg, 2000). In contrast, the A-class ODNs strongly stimulate pDCs to secrete IFN-α/-β, but are poor at activating B-cells. C-class ODNs combine properties of the A and B classes, and are strong inducers of interferon-α and thus activation of NK and maturation of DC. In our experiments, Type A CpG was the most effective against HSV-2 and provided 97% reduction in viral titres while CpG B and C provided 70% and 76% reduction in HSV-2 titers, respectively (Fig. 2B). Interestingly, non-CpG control ODN for Type A was also able to reduce HSV-2 replication, although to a significantly lesser extent.

3.3. Anti-viral activity in GEC supernatants following TLR treatment

In order to examine the induction of anti-viral response in GEC following TLR treatment, we used a VSV-GFP assay which assesses the presence of biologically active IFN (Meager, 2002). Using this assay, we measured the ability of VSV-GFP to replicate in the presence of supernatants from TLR ligand-treated epithelial cultures. Poly I:C treatment of endometrial ECs induced highest amounts of anti-viral activity in the GEC supernatants among all the TLR ligands and inhibited >98% VSV-GFP infection (Fig. 3A). The anti-viral activity in supernatants from LPS-treated monolayers was comparable to untreated monolayers. Other TLR ligands induced intermediate levels of anti-viral activity in GEC supernatants, inhibiting between 25% and 60% VSV-GFP infection. The protection conferred

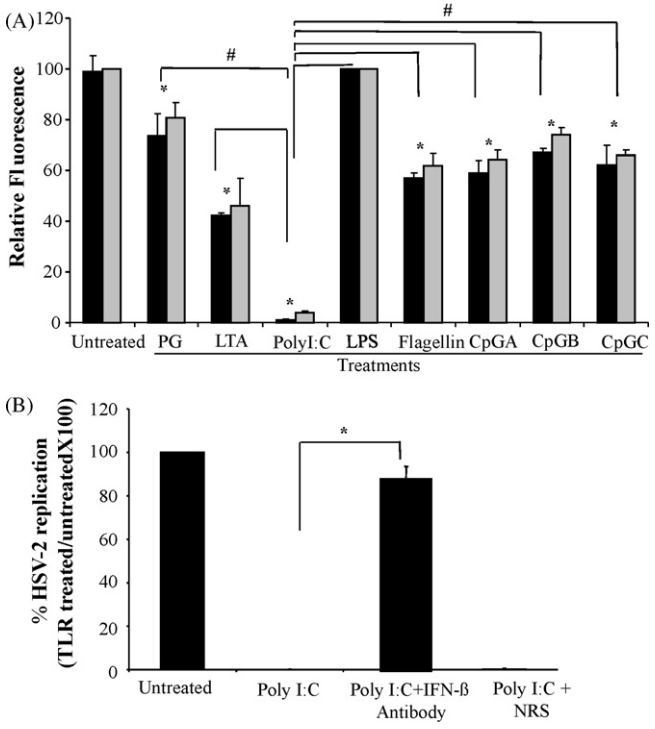

Fig. 3. Interferon production by GECs in response to TLR ligand treatment, measured by VSV assay. (A) Biologically active interferon was measured in supernatants collected after 24 h of TLR ligand treatment from endometrial epithelial monolayers by VSV-GFP assay. Fluorescence values of TLR ligand-treated cultures were compared with control cultures containing no interferon and expressed as relative fluorescence. The amount of relative fluorescence of VSV-GFP shown in the graph is inversely proportional to the amount of biologically active interferon in supernatants. Black bars indicate interferon in apical supernatants and grey bars indicate basolateral supernatants of triplicate cultures from three to seven different GEC cultures. The amount of interferon produced in supernatants collected after TLR ligand treatment was significantly higher than untreated controls ($^*p < 0.001$) with the exception of LPS. Poly I:C treatment increased interferon production significantly higher than all other ligand treatments ($^\#p < 0.001$). (B) Endometrial epithelial monolayers were treated with Poly I:C (25 μg/ml) alone or with IFN-β neutralizing antiserum or with normal rabbit serum for 24 h. Each treatment group was run in triplicate wells for each GEC culture. Treated and untreated (medium treated) control monolayers were infected with HSV-2, 333 (10^4 PFU/well). The reduction in viral titers in treated, infected monlayers was compared with untreated, infected controls and plotted as percent of untreated sample. Viral counts were reduced by >99% following treatment with Poly I:C compared to untreated controls. Addition of normal rabbit serum did not affect the ability of Poly I:C in reducing viral counts but neutralization of IFN-β production by Poly I:C treatment by the addition of IFN-β-specific antibodies resulted in significantly higher viral counts compared to Poly I:C-treated monolayers ($p < 0.00001$). Data shown is representative of one of two separate experiments with similar results.

against VSV-GFP by transfer of supernatants indicated the presence of biologically active IFN present in TLR ligand-treated GEC cultures.

Since IFN-β has been shown to be the main mediator of anti-viral effects, we next decided to assess the contribution of IFN-β in the anti-viral effect seen in TLR ligand-treated GEC supernatants. GEC monolayers were treated with Poly I:C alone, with Poly I:C + rabbit polyclonal anti-IFN-β antiserum or Poly I:C + normal rabbit serum for 24 h. Poly I:C treated and control cultures (Poly I:C untreated) were infected with HSV-2. 24 h post-infection, apical and basolateral supernatants were collected and HSV-2 titers were measured in the supernatants by vero plaque forming assays. Viral replication was completely inhibited in GEC cultures treated with Poly I:C, compared to untreated cultures (Fig. 3B). Addition of anti-IFN-β antibody reduced the ability of Poly I:C-treated GECs to inhibit HSV-2 replication by 90% ($p < 0.00001$). In contrast, normal rabbit serum did not have any effect on inhibition of HSV-2 replication by Poly I:C-treated GEC cultures. When supernatants from the Poly I:C and Poly I:C + IFN-β antibody-treated cultures were tested in VSV-GFP assays, similar results were obtained. Supernatants from Poly I:C and Poly I:C + normal rabbit serum-treated GEC cultures completely inhibited VSV-GFP infection, whereas supernatants from Poly I:C + IFN-β-treated GEC cultures showed only 10–20% inhibition (data not shown). These results showed that treatment of GECs with Poly I:C resulted in production of IFN-β and this was the main mediator in inhibition of HSV-2 replication.

3.4. Nitric oxide (NO) production by GECs following TLR treatment

We next examined NO production by GEC monolayers, since it has been shown to be also a key anti-viral response (Colasanti and Suzuki, 2000). Nitric oxide was measured by Griess assay in GEC supernatants collected after 24 h (data not shown) and 48 h (Fig. 4) after ligand treatment. Supernatants collected from medium-treated and LPS-treated cervical epithelial cultures had undetectable amounts of NO. Other ligands initiated NO production in both endometrial and cervical epithelial cultures within 24 h; maximum accumulated levels in supernatants were observed at 48 h post-treatment. Maximum NO production was seen in Poly I:C-treated endometrial cultures and cervical cultures (Fig. 4A and B).

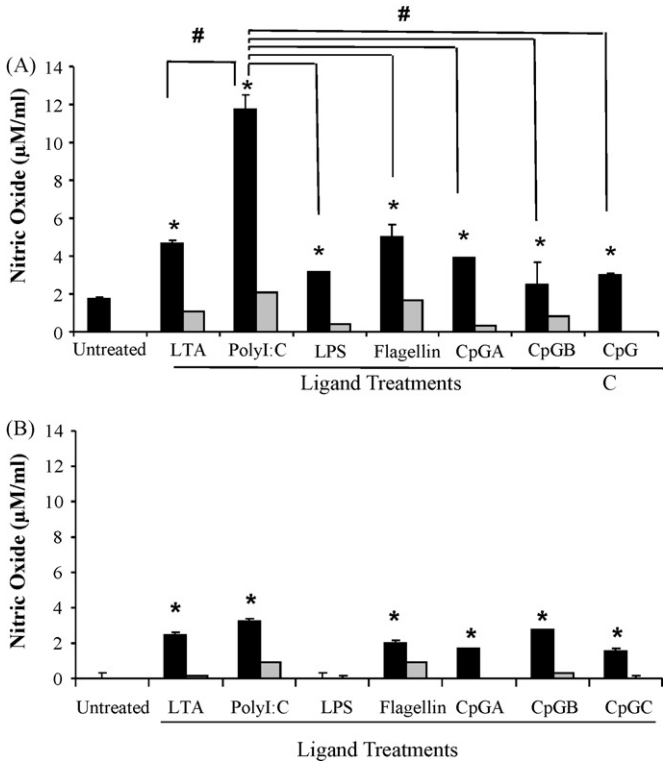

Fig. 4. Production of nitric oxide by GECs in response to TLR ligand treatment. Nitric oxide production was measured by Griess assay in supernatants collected from cultured primary female genital epithelial cells 48 h post-treatment with TLR ligands in endometrial (Fig. 5A) and cervical epithelial cells (Fig. 5B). Black bars indicate the supernatants collected from apical side of the monlayers and grey bars indicate the supernatants collected from the basolateral side of the monolayers. Each treatment group was run in triplicate wells for each GEC culture and data shown is from three different GEC cultures. Endometrial epithelial cells produced significantly higher amounts of nitric oxide in the supernatants 48 h after TLR ligand treatments compared to untreated controls ($p < 0.001$). Poly I:C treatment produced significantly high amounts of nitric oxide compared to other TLR ligand treatments. In cervical cells all the ligand treatments significantly increased amounts of nitric oxide except LPS when compared with untreated control ($p < 0.001$).

3.5. Induction of inflammatory cytokines by GECs following TLR treatment

Other studies have shown cytokine production by GECs in response to selected TLR ligands (Andersen et al., 2006; Schaefer et al., 2005). We decided to compare the production of pro-inflammatory cytokines in GEC following treatment with eight different TLRs used in this study. Following 24 and 48 h of ligand treatment, supernatants from GECs were collected and analyzed for 6 different cytokines including IL-6, IL-8, and TNF-α, IL-1β, IL-10 and IL-12. Similar profile of cytokine production was seen at both 24-h (not shown) and 48-h (Fig. 5A–D) time points, although the overall cytokine accumulation in supernatants was higher at 48 h compared to 24 h.

Both cervical and endometrial epithelial cells constitutively produced high quantities of IL-6, secreted preferentially on apical side of the polarized epithelium. Poly I:C increased IL-6 levels significantly on the basolateral side of both endometrial and cervical monolayers. IL-8 was also produced in high amounts by both types of epithelium; however there was no preferential secretion. There was significant increase in IL-8 production after CpGB (cervical and endometrial) and CpGC (endometrial) treatment. TNF-α was increased significantly in apical supernatants of Poly I:C-treated endometrial epithelial monolayers compared to supernatants from untreated cultures. TNF-α was also increased in Poly I:C and CpG B and C treated cervical ECs. However, the fold increase was not as high as that seen in endometrial cells. Cervical and endometrial epithelial cells constitutively produced IL-1β on the apical side. Poly I:C treatment significantly increased apical secretion of IL-1β in endometrial culture supernatants. No consistently significant changes were noted in the other two cytokines tested (IL-10 and IL-12).

3.6. Activation of transcription factors in GECs following TLR ligand treatment

Since we saw significant production of inflammatory cytokines as well as IFN-β following Poly I:C treatment, we next examined the intracellular pathways involved in induction of inflammatory cytokines and Type I interferon. Poly I:C is a TLR3 ligand and TLR3 activation has been shown to activate both Myd88-dependent and independent pathways (Alexopoulou et al., 2001; Kawai and Akira, 2007a). We first examined NFκB to examine the activation of Myd88-dependent pathway in Poly I:C-treated GEC cultures. Primary endometrial cultures were treated with Poly I:C for 2,

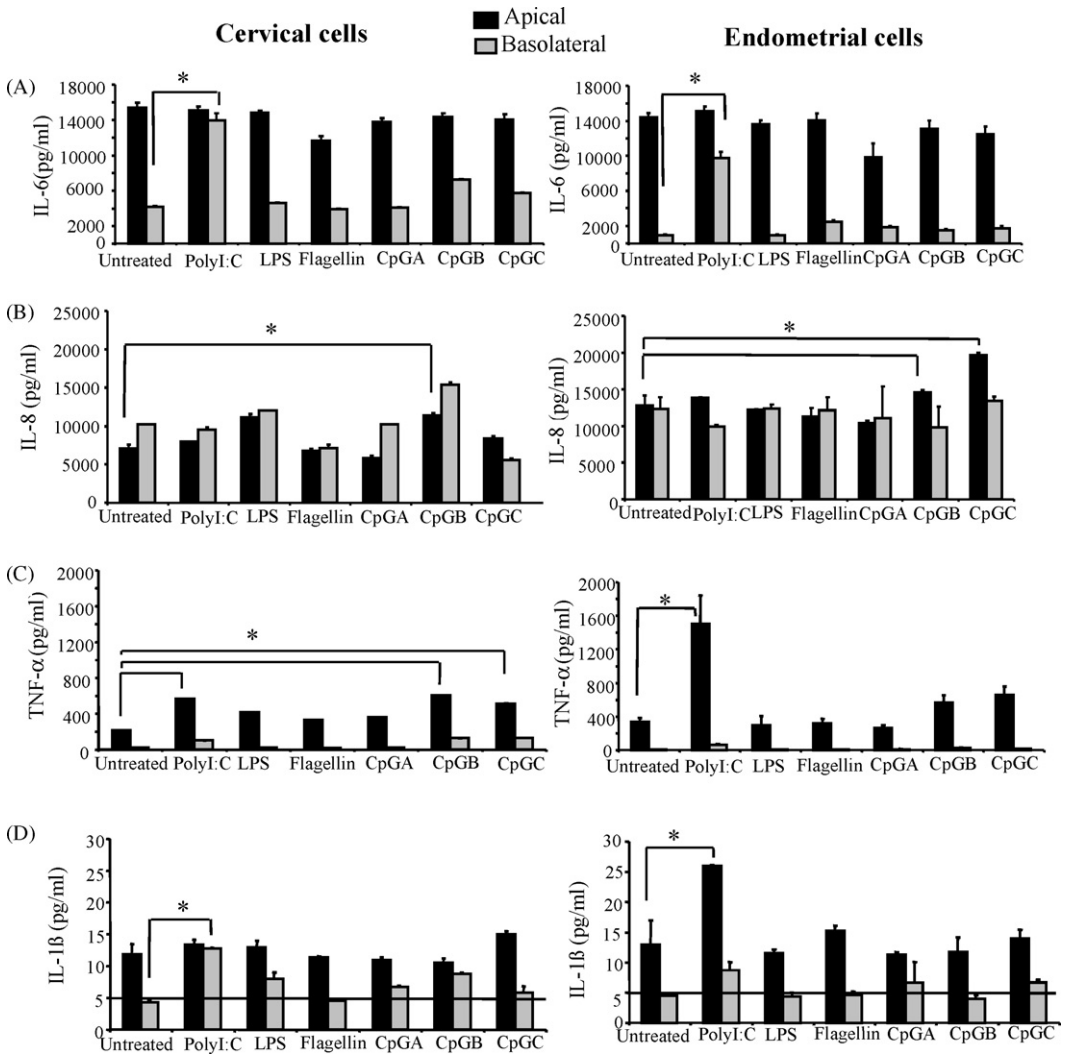

Fig. 5. Cytokine production by genital epithelial cells following TLR ligand treatment. Supernatants were collected from endometrial and cervical epithelial cells 24-h (not shown) and 48-h post-TLR ligand treatment and assayed by Luminex multi-analyte kit for the following cytokines: (A) IL-6; (B) IL-8; (C) TNF-α; (D) IL-1B. Black bars indicate the supernatants collected from apical side of the monlayers and grey bars indicate the supernatants collected from the basolateral side of the monolayers. Significant differences ($p < 0.001$, *t*-test) between specific treatments are indicated in each panel. Triplicate wells were run for each different treatment. The figure is representative of two separate experiments with similar results.

4 and 8 h or treated with media as control. As a negative control, cultures were also treated with LPS. Cultures were fixed and stained for NFκB and nuclear translocation was examined by confocal microscopy. Maximum NFκB nuclear translocation was seen in EC cultures at 2 h (Fig. 6A) post-treatment, compared to 4 and 8 h (not shown). No nuclear translocation was seen in LPS-treated cultures (not shown). We further investigated if Poly I:C treatment of GECs activated Myd88-independent pathway of anti-viral activity. GEC cultures were treated with Poly I:C for 2, 4 and 8 h and examined for IRF-3 activation. IRF-3 is one of the key transcription factors in the Myd88-independent pathways (Kawai and Akira, 2007a). Sendai virus infection has been shown to induce IRF-3 activation in a number of cell lines (Elco et al., 2005). Therefore GEC cultures were infected with Sendai virus and used as a positive control for IRF-3 nuclear translocation. Clear nuclear translocation of IRF-3 was seen at 2 h Poly I:C-treated GEC cultures (Fig. 6B). Translocation of IRF-3 was seen in some but not all cells and was comparable to that seen in Sendai virus infected cultures. The results show that following Poly I:C treatment, both Myd88-dependent and independent pathways of anti-viral activity were activated in GECs.

4. Discussion

The present study provides direct evidence that activation of anti-viral responses by TLR ligands lead to decreased replication of HSV-2 in primary genital epithelial cells. Treatment of epithelial monolayers with TLR3 ligand Poly I:C, TLR9 ligand CpG A and TLR5 ligand flagellin led to greatest reduction in HSV-2 replication. This anti-viral activity correlated closely with biological interferon activity and NO production in supernatants of treated GECs. Antibody neutralization experiments indicated that in Poly I:C-treated GEC cultures, IFN-β was predominantly responsible for the anti-viral activity. Of the different TLR ligands tested, Poly I:C treatment also led to enhanced production of inflammatory cytokines IL-1β, IL-6 and TNF-α in endometrial epithelial cells. TLR4 ligand, LPS, on the other hand failed to have any effect on HSV-2 replication. Coincident with that, interferon activity and NO production was absent in supernatants from LPS-treated GEC cultures, nor was there induction of any inflammatory cytokines. All other TLR ligands provided intermediate levels of protection (50–97%) against HSV-2. This correlated with intermediate interferon activity in supernatants of treated

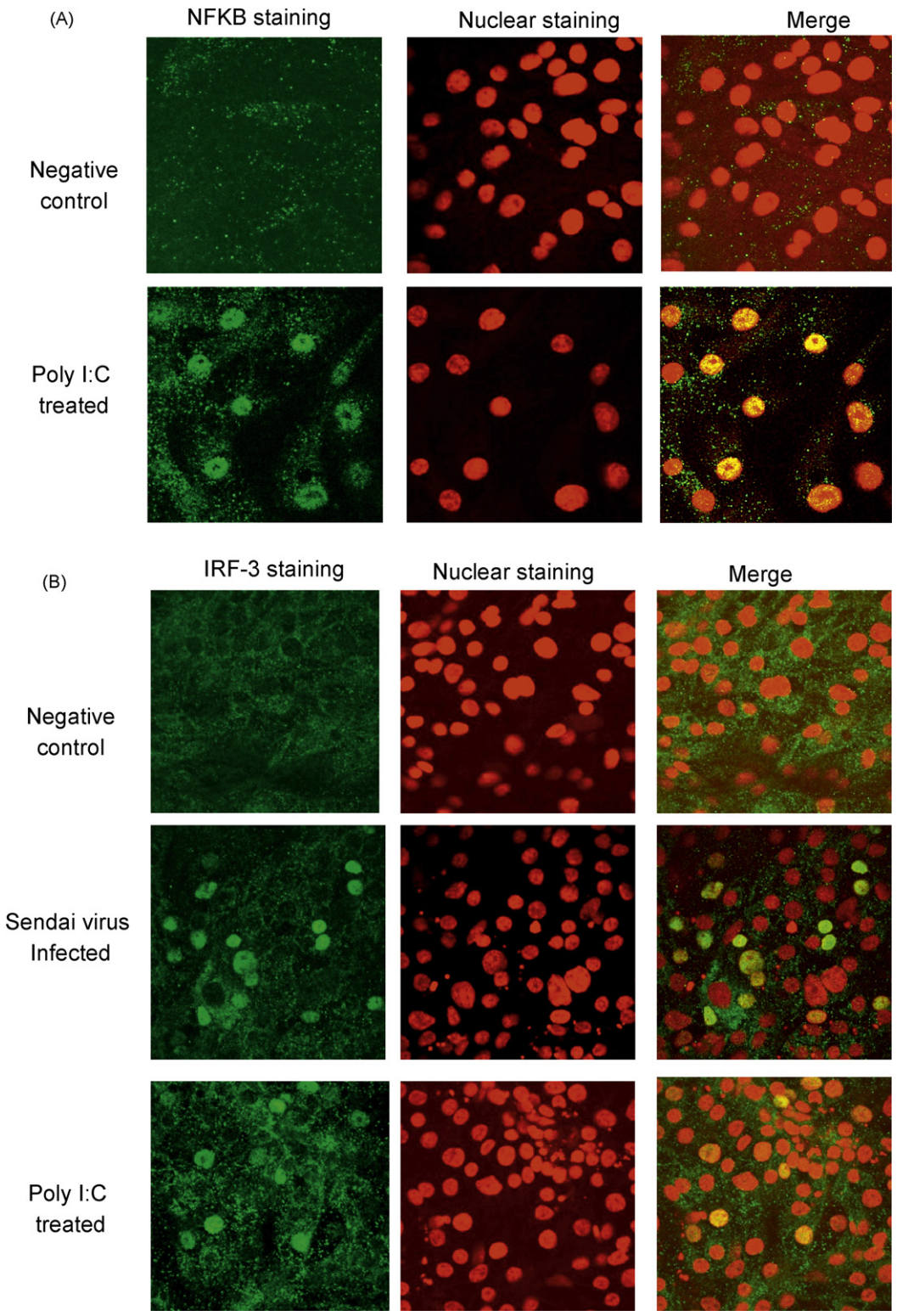

Fig. 6. Activation and translocation of NFκB and IRF-3 in genital epithelial cells following Poly I:C treatment. Primary female endometrial epithelial monolayers were treated with Poly I:C for 2 h and stained for NFκB (A) and IRF-3 (B). Sendai virus infection was used as positive control for IRF-3 staining. Nuclear staining, seen in red, by propidium iodide. Magnification 1260×. Images were captured by laser-scanning confocal microscope. Representative images are shown from one of two separate experiments with similar results.

cultures. Further investigation of the pathways by which Poly I:C may be inducing inflammatory cytokines and interferon indicated both Myd88-dependent activation of NFkB as well as IRF-3 activation indicating potential activation of Myd88-independent pathway. The results from these studies provide support for examining the potential use of TLR ligands as topical microbicides to protect against sexually transmitted viruses, such as HSV-2.

Previous studies have demonstrated efficacy of anti-viral protection induced by TLR ligands in *in vivo* mouse models (Ashkar et al., 2003, 2004; Harandi et al., 2003; Herbst-Kralovetz and Pyles, 2006b). Other studies have demonstrated production of anti-viral factors and cytokines by genital epithelial cell lines and primary cells, but not demonstrated the efficacy of these responses against a specific viral/bacterial pathogen (Andersen et al., 2006; Fichorova et al., 2002; Schaefer et al., 2005). A recent study demonstrated that treatment with TLR3/TLR9 ligand but not TLR4 ligand was effective in improving the outcome of HSV-1 encephalitis in mice (Boivin et al., 2008). The present study directly determined the anti-viral activity against a sexually transmitted virus, HSV-2. The results show that the human primary GEC culture system is quite suitable for testing the potential efficacy of candidate microbicides against viral pathogens. Addition of different TLR ligands led to varying, but reproducible, decrease in HSV-2 replication, thereby indicating distinct response of the epithelial cell monolayers to each ligand. Given that this culture system uses primary genital epithelial cultures grown from tissues of women, we believe these studies provide a clinically relevant system to test microbicides and anti-viral formulations.

One of the historical and ongoing controversies surrounding microbicides is their unpredictability in induction of local inflammation in the genital tract (van de Wijgert and Shattock, 2007). Previous trials on Nonoxynol-9 ended when increased HIV-1 infection was seen, likely due to local inflammation (Hillier et al., 2005). Recent trials on other promising candidate, cellulose sulphate, were also recently terminated due to increased number of HIV infections seen in treated groups (van de Wijgert and Shattock, 2007). While previous studies have been able to predict the efficacy of microbicides against pathogens, they failed to envisage the inflammatory potential of these formulations. Our results indicate that the ex vivo GEC culture system can predict potential of local inflammation, since we were able to measure inflammatory cytokines produced by GECs following treatment with different TLRs. This provides an additional advantage for testing microbicide candidates in this system. Based on our current results, we predict that while TLR3 ligand, Poly I:C, could provide greatest protection against HSV-2, it could also induce production of inflammatory cytokines by the epithelial cells. Inflammatory cytokine induction by Poly I:C and other TLR ligands in the genital tract has the potential to enhance susceptibility to HIV via recruitment of target cells (Miller and Shattock, 2003). Additionally, inflammatory cytokines such as TNF-α are known to enhance HIV replication (Kedzierska et al., 2003). Therefore caution needs to be exercised in proposing Poly I:C or other TLR ligands, as promising microbicide candidates.

In previous studies, TLR3 and 9 ligands, Poly I:C and CpG, respectively, demonstrated complete protection in mouse models of genital HSV-2 infection whereas TLR4 ligand, LPS did not show any protection (Ashkar et al., 2003, 2004). The mechanism of protection was shown to be mediated by IFN-β production (Gill et al., 2006). However, it was unclear from these studies which cells in the genital tract were primarily responsible for the protective effect. The results from the present study show that protective effect of TLR ligands could possibly be due to direct effect of these ligands on genital epithelial cells. Similar to in vivo studies, both TLR3 ligand Poly I:C and TLR9 ligand, CpG induced production of biologically active interferon that correlated with level of protection. Interestingly, the in vivo studies showed significant systemic inflammatory responses in CpG-treated mice while no such effects were noted in Poly I:C-treated mice. In the present study, human genital epithelial cells responded to Poly I:C treatment by production of significant amounts of inflammatory cytokines, TNF-α, IL-1β and IL-6. Whether these differences in response are due to species-specific effects, is not clear. Another possibility is that in the current study we have only examined effect of TLR ligands on epithelial cells, whereas in vivo the responses are outcome of complex interactions between epithelial cells and other cells that reside in the genital tract such as Langerhans cells, tissue fibroblasts and immune cells. Nevertheless, the differences emphasize the need for caution in extrapolating data from mouse studies to clinical setting. While we would argue that results from our studies are more relevant clinically since the ex vivo cultures are from human tissue, parallel clinical studies need to be conducted to confirm that the results from ex vivo culture studies are better at predicting the outcomes in humans, compared to in vivo data from mice studies.

Little is known about TLR ligand induced signaling in primary epithelial cells. Most studies done on TLRs in cell lines show that signaling pathways initiated by TLR ligands are regulated by adaptor protein Myd88 which leads to activation of NFκB and subsequent upregulation of inflammatory cytokines (Barton and Medzhitov, 2003; Kawai and Akira, 2007a,b). TLR3 does not require Myd88 but requires adaptor molecule TRIF which then leads to IRF-3 activation and subsequent upregulation of IFN-β (Alexopoulou et al., 2001). Our results clearly show that TLR3 ligand, Poly I:C can induce activation of both NFκB and IRF-3 and subsequent production of both interferon as well as pro-inflammatory cytokines in primary GECs. The innate responses seen in these primary cultures may be more representative of normal innate responses in mucosal epithelium.

In conclusion, this study provides direct evidence that female genital epithelial cells can be directly activated by TLR ligands to induce an innate anti-viral state that can provide protection against sexually transmitted viruses, such as HSV-2. This provides a good rationale to test whether this mechanism could provide a natural way to boost the innate defense of genital epithelial against sexually transmitted infection, in the form of a microbicide. Both bacterial and viral TLR ligands were able to protect against HSV-2, indicating that such a formulation would be useful for protection against both types of pathogens. However, the results need to be further examined with cautious optimism since inflammatory cytokines were also induced by some of the TLR ligands.

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