Endometrial Epithelial Cell Responses to Coinfecting Viral and Bacterial Pathogens in the Genital Tract Can Activate the HIV-1 LTR in an NFκB-and AP-1–Dependent Manner

Victor H. Ferreira,[1] Aisha Nazli,[1] Ghaznia Khan,[1] M. Firoz Mian,[1] Ali A. Ashkar,[1] Scott Gray-Owen,[2] Rupert Kaul,[3] and Charu Kaushic[1]

[1]Department of Pathology and Molecular Medicine, Centre for Gene Therapeutics, Michael DeGroote Centre for Learning and Discovery, McMaster University, Hamilton, [2]Department of Medical Genetics and Microbiology, and [3]Clinical Sciences Division, Department of Medicine, University of Toronto, Ontario, Canada

Background. Sexually transmitted infections (STIs) are associated with increased human immunodeficiency virus type 1 (HIV-1) susceptibility and viral shedding in the genital tract, but the mechanisms underlying this association are poorly understood.

Methods. Direct activation of HIV long terminal repeats (LTRs), a proxy measure for HIV-1 replication, was measured after treatment of 1G5 T cells with Toll-like receptor (TLR) ligands, herpes simplex virus type 1 or 2 (HSV-1/2), or *Neisseria gonorrhoeae*. For indirect activation, 1G5 T cells were incubated with supernatants from female primary genital epithelial cells (GECs) previously exposed to these agents. Proinflammatory cytokines and chemokines were measured in GEC supernatants. Proinflammatory pathways were blocked to determine the mechanisms of direct and indirect HIV-LTR activation.

Results. HSV-1/2, *N. gonorrhoeae,* and TLR ligands FimH (TLR-4), flagellin (TLR-5), and Poly (I:C) (TLR-3) directly induced HIV-LTR activation in 1G5 T cells. Supernatants collected from GECs incubated with these agents indirectly induced HIV-LTR activation. Production of tumor necrosis factor α, interleukin 6, interleukin 8, and monocyte chemoattractant protein-1 was elevated in GECs exposed to copathogens. Inhibition of nuclear factor κB and activator protein-1 (AP-1) signaling pathways in 1G5 T cells abrogated both direct and indirect HIV-LTR activation.

Conclusions. STIs may increase HIV-1 replication in the female genital tract via proinflammatory signaling pathways directly and indirectly via their effects on GECs. This increased HIV-1 replication may enhance sexual and vertical HIV transmission.

Heterologous infection with viruses, bacteria, and other organisms, such as protozoan parasites, may significantly influence the clinical course of human immunodeficiency virus (HIV) infection, as well as the acquisition, replication, and transmission of the virus [1–3]. UNAIDS statistics indicate that women constitute >50% of HIV-infected individuals globally [4]. Women also carry a disproportionately higher burden of sexually transmitted infections (STIs), compared with men [5]. Sexually transmitted pathogens, such as *Neisseria gonorrhoeae* and herpes simplex virus (HSV), may play an important role in increasing HIV replication in the female genital tract. *N. gonorrhoeae* infection is among the most common bacterial STIs in people living with HIV/AIDS [6]. Symptomatic gonococcal infection is associated with increased detection rates of HIV DNA in the urogenital tract [7–10]. With prevalence rates ranging from 10% to 60%, genital herpes, a chronic viral infection, poses a substantial burden on people living

Received 6 December 2010; accepted 4 March 2011.

Potential conflicts of interest: none reported.

Presented in part: Keystone Symposia: HIV Biology and Pathogenesis; Sante Fe, New Mexico; 12–17 January 2010. Abstract 202; 23rd Canadian Society for Immunology Conference; Niagara Falls, Ontario; 23–26 April 2010. Abstract 131.

Correspondence: Charu Kaushic, PhD, Dept of Pathology and Molecular Medicine, Centre for Gene Therapeutics, Michael DeGroote Centre for Learning and Discovery, Rm 4014, McMaster University, 1200 Main St West, Hamilton, ON L8N 3Z5, Canada (kaushic@mcmaster.ca).

The Journal of Infectious Diseases 2011;204:299–308

0022-1899 (print)/1537-6613 (online)/2011/2042-0020$14.00
DOI: 10.1093/infdis/jir260

with HIV/AIDS [11]. Genital herpes is primarily caused by HSV-2, although HSV-1 infection is increasingly being confirmed in cases of primary genital herpes [11–13]. Clinical studies show that HSV-2 reactivation is frequently accompanied by an increase in HIV plasma viral load and conversely, treatment of HSV-2 is seen to slow down progression of HIV [14–16].

Heterosexual transmission across genital tissue is the primary route by which women acquire HIV [17]. Sexually transmitted copathogens may be present in the female genital tract prior to, during, or following HIV exposure. However, little is known about the mechanism by which these coinfections may enhance HIV replication in the female genital tract. In HIV-infected women, T cells in the female genital tract may come into direct contact with the coinfecting pathogens, resulting in activation of the 5'-HIV long terminal repeat (LTR) region, resulting in enhanced HIV replication. Alternatively, proinflammatory cytokines present in the genital tract may indirectly enhance HIV replication in infected T cells.

Genital epithelial cells (GECs) are the first cells in the female genital tract to encounter sexually transmitted pathogens. In response to interactions with microbes, GECs have been shown to produce proinflammatory cytokines, including tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6), which can increase HIV replication [18, 19]. Therefore, HIV replication may be upregulated in infected cells exposed to proinflammatory cytokines produced by GECs, both during initial exposure and after established infection. This possibility is supported by our recent study showing that exposure of GECs to HIV upregulated the production of TNF-α, leading to impairment of the mucosal epithelial barrier in the genital and intestinal tract and thus facilitating viral and bacterial translocation [20].

The current study was designed to examine whether sexually transmitted coinfections were capable of enhancing HIV replication in the female genital microenvironment. We found that viral and bacterial Toll-like receptor (TLR) ligands, as well as HSV-1, HSV-2, and *N. gonorrhoeae,* were capable of directly driving the 5'-LTR promoter in transfected T cells in a mechanism dependent on both nuclear factor κB (NFκB) and activator protein-1 (AP-1). Furthermore, in response to bacterial TLR ligands, as well as infection by HSV-1, HSV-2, or *N. gonorrhoeae*, GECs secreted immune factors that could indirectly activate the HIV-LTR, in a mechanism also dependent on NFκB and AP-1.

MATERIALS AND METHODS

Pathogens and Cell Lines

Vero cells (ATCC) were maintained in minimum essential medium-α (α-MEM) supplemented with 5% fetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin/streptomycin (Sigma-Aldrich), 2 μM L-glutamine (Invitrogen), and 10 μM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). HSV-1 KOS and HSV-2 333 viral stocks were prepared by infecting Vero cells at a multiplicity of infection of 0.01 for 24–48 hours. Infected cells were harvested and disrupted by 3 cycles of freeze-thaw. Preparations were spun down at 1500 rpm, and supernatants were stored at −80°C. Viral titration of stocks was accomplished by serially diluting the stock in α-MEM and performing standard Vero plaque assay [21]. *N. gonorrhoeae* clinical strain 2071 was grown at 37°C in a 5% CO_2 humidified incubator from frozen stocks on GC agar base supplemented with 1% (vol/vol) IsoVitaleX enrichment (Becton, Dickinson-DIFCO) as described elsewhere [22]. 1G5 Jurkat T cells were maintained in Roswell Park Memorial Institute media supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 2 μM L-glutamine, and 10 μM of HEPES.

Source of Genital Tissue and Epithelial Cell Preparation

Female genital tract tissues were obtained from women aged 34–56 years (mean, 44.8 ± 6.7 years) undergoing hysterectomies for nonmalignant gynecological purposes at McMaster University Medical Centre in Hamilton, Ontario, Canada. Written informed consent was received in accordance with the approval of the Hamilton Health Sciences–McMaster University Research Ethics Board. The most common reasons for surgery were uterine fibroids or heavy bleeding. Uterine tissues were used only if free of malignancy as determined by clinical pathologists. A small piece of the epithelium that was not in the vicinity of the fibroids was excised for processing. The protocol for isolation and culture and details regarding the purity of primary GECs has been described elsewhere [20, 23, 24]. Briefly, endometrial tissues were minced into small pieces and digested enzymatically, and epithelial cells were isolated by means of a series of separations through nylon mesh filters of different pore sizes (Small Parts, Inc). GECs were seeded onto and grown on Matrigel (Becton, Dickinson) coated, 0.4-μm pore-size polycarbonate membrane tissue culture inserts (BD Falcon) with primary epithelial cell culture medium [24]. Polarized monolayers were formed within 5–7 days. The confluency of GEC cultures was monitored by means of transepithelial resistance across polarized monolayers measured by a volt-ohm meter (World Precision Instruments) and were considered confluent when transepithelial resistance values reached >1000 Ω/cm.

TLR Ligands

The TLR-2 ligands lipoteichoic acid (LTA) from *Staphylococcus aureus* and peptidoglycan from *S. aureus,* the TLR-3 ligand polyinosinic:polycytidylic acid (Poly [I:C]), and TLR-4 ligand lipopolysaccharide from *Escherichia coli* 026:B6 were purchased from Sigma-Aldrich. FimH, a novel TLR-4 ligand [25, 26], and flagellin, a TLR-5 ligand, were prepared at McMaster University. The TLR-7 ligand loxoribine was purchased from Invivogen. CpG-oligonucleotides (TLR-9 ligands) were prepared by Mobix Facility at McMaster University, and sequences for CpG-A-2216, CpG-B-2006, and CpG-C-2395 have been published elsewhere [23]. All

ligands were diluted to a final concentration of 10 μg/mL, with the exception of Poly (I:C) and loxoribine, which were prepared to 25 and 339 μg/mL concentrations, respectively.

Cytokine/Chemokine Measurement

Apical and basolateral supernatants were analyzed for multiple cytokines using the Luminex multianalyte technology (Millipore) as previously described [27]. Multiplex bead-based sandwich immunoassay kits were used to measure levels of interleukin 1α (IL-1α), IL-6, interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), granulocyte-macrophage colony stimulating factor (GM-CSF), regulated on activation normal T cell expressed and secreted (RANTES), and TNF-α in accordance with the manufacturer's instructions.

Direct Activation of the HIV-LTR

To measure direct activation of the HIV-LTR in 1G5 cells by TLR ligands or intact pathogens, 1×10^6 1G5 cells were treated with TLR ligands for 24 hours at 37°C or incubated with 10^4 PFUs of HSV-1 KOS, HSV-2 333, or 10^6 colony-forming units (CFUs) of *N. gonorrhoeae* strain 2071 for 16 hours at 37°C, after which the cells were lysed and luciferase activity was determined using a luciferase assay (Agilent) in accordance with the kit's instructions. Control cultures included either 1G5 Jurkat T cells incubated with media alone or mock-treatment with uninfected Vero cell lysate (for HSV-1/2) or washing collected from GC agar plates (for *N. gonorrhoeae*).

Indirect Activation of the HIV-LTR

To measure indirect activation of the HIV-LTR in 1G5 cells by TLR ligands, confluent primary endometrial GECs were treated with TLR ligands for 2 hours at 37°C. The cells were washed 5 times with 1x phosphate-buffered saline (PBS) and replenished with fresh media and incubated at 37°C. Then 24-hour post-treatment apical and basolateral supernatants were collected. At this point, 100 μL of apical and basolateral supernatants were added to 1×10^6 1G5 cells for 24 hours at 37°C, after which the cells were lysed and luciferase activity was determined. To measure indirect HIV-LTR activation by HSV-1, HSV-2, or *N. gonorrhoeae*, confluent primary GECs were infected with 10^4 PFUs of HSV-1 KOS or HSV-2 333 or 10^6 CFUs of *N. gonorrhoeae* strain 2071 for 2 hours, washed 5 times, and supernatants were collected from the apical and basolateral chambers. Supernatants collected from HSV-infected cells were exposed to 1 cycle of 10 kJ of UV energy using a Stratalinker (Agilent) to ensure that there was no residual live virus. Supernatants collected from *N. gonorrhoeae*–infected GECs were filter sterilized using a 0.2-μm filter (BD Falcon) to remove any residual bacteria. The supernatants were then incubated with 1×10^6 1G5 cells for 24 hours, and luciferase activity was measured. Mock infected controls consisting of supernatants collected from primary GECs incubated with uninfected Vero cell lysate (for HSV-1/2) or a washing from GC agar plates (for *N. gonorrhoeae*) were also included, in addition to media controls.

Inhibition of Intracellular Pathways

In order to block anti-inflammatory pathways, 1G5 cells were pretreated with 5 μM curcumin (Sigma-Aldrich), 10 μM NFκB inhibitor pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich), p38 MAP kinase inhibitor SB203580 (Invivogen), or c-Jun N-terminal kinase (JNK) inhibitor SP600125 (Invivogen) for 1 hour, after which the cells were washed with 1xPBS before being exposed to direct or indirect treatments.

Statistical Analysis

GraphPad Prism version 4 (GraphPad Software) was used to compare 3 or more means by 2-way analysis of variance. When an overall statistically significant difference was seen ($P < .05$), posttests were performed to compare pairs of treatments, using the Bonferroni posttest method to adjust the *P*-value for multiple comparisons.

RESULTS

TLR Ligands Can Directly and Indirectly Drive HIV-LTR Activation in 1G5 Cells

To examine how HIV coinfecting pathogens in the genital tract may play a role in increasing HIV replication, we tested whether TLR ligands representative of viral and bacterial pathogens, were capable of directly or indirectly *trans*-activating the HIV-LTR in 1G5 Jurkat T cells that expressed the luciferase gene under the control of the HIV-LTR promoter [28]. For direct HIV-LTR activation to occur, a ligand would directly interact with its cognate receptor, causing an intracellular cascade resulting in HIV-LTR activation. 1G5 cells treated with Poly (I:C) (TLR-3 ligand) ($P < .0403$), the Gram-negative bacteria fimbriae protein FimH (TLR-4 ligand) ($P < .001$) and the bacterial flagella protein flagellin (TLR-5 ligand) ($P < .001$) induced potent HIV-LTR activation in comparison to mock treatment (Figure 1a). Peptidoglycan (TLR-2 ligand), lipoteichoic acid (TLR-2 ligand), lipopolysaccharide (TLR-4 ligand), loxoribine (TLR-7 ligand) and CpG oligonucleotides (TLR-9) did not directly induce HIV-LTR activation in 1G5 cells.

In order to examine indirect activation of HIV-LTR in 1G5 T cells, we tested whether TLR ligands could induce GECs to secrete factors that could induce indirect activation of the HIV-LTR. 1G5 cells were incubated with apical or basolateral cell culture supernatants from TLR ligand treated primary endometrial GECs. Both apical (Figure 1b) and basolateral (Figure 1c) supernatants collected from FimH- and flagellin-treated GECs increased HIV-LTR activation in comparison to supernatants collected from primary cells that had been mock-treated. To rule out the possibility that the indirect activation could be due to TLR ligand contamination in primary GEC supernatants, PBS washes

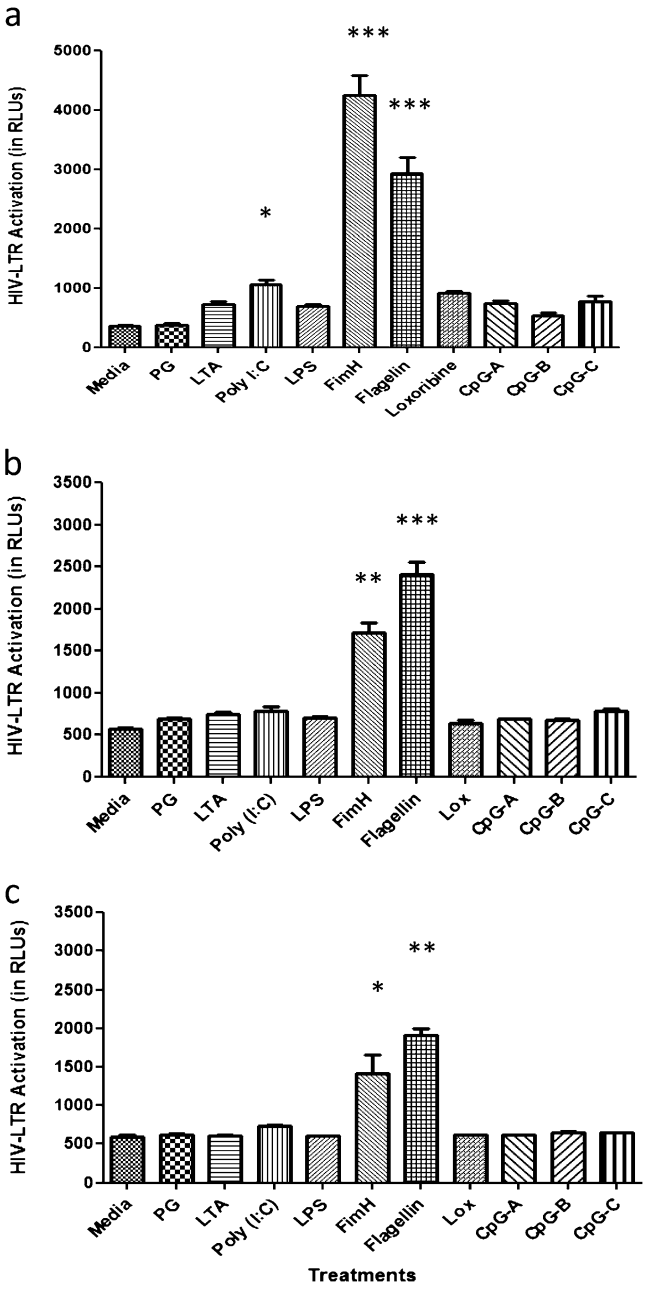

Figure 1. Toll-like receptor (TLR) ligands can directly and indirectly activate the human immunodeficiency virus (HIV)–long terminal repeat (LTR) in 1G5 cells. To assess whether coinfections directly contribute to HIV-LTR activation (*A*), 1×10^6 1G5 Jurkat T cells expressing the luciferase gene under the control of the HIV-LTR promoter were treated with peptidoglycan, LTA, Poly (I:C), lipopolysaccharide, FimH, flagellin, loxoribine, or CpG-oligonucleotides for 24 hours. After this, the cells were lysed, and luciferase activity was measured and reported in relative light units. Data are mean ± standard error of the mean (SEM) of 4 separate experiments. To assess whether coinfections in the female genital tract could indirectly contribute to HIV-LTR activation, primary endometrial genital epithelial cell cultures were grown to confluency on transwell inserts and treated with the same TLR ligands described above for 2 hours. Treatments were removed and the cells were washed 5 times with 1x phosphate-buffered saline and subsequently replenished with fresh media. Then, 24 hours after treatment, apical (*B*) and basolateral (*C*) supernatants were collected and incubated with 1×10^6 1G5 cells for 24 hours, after which the cells were lysed, and luciferase activity was measured. Data are mean ± SEM of 3 separate experiments. An asterisk indicates $P < .05$, a double asterisk indicates $P < .01$, and a triple asterisk indicates, $P < .001$; RLU, relative light unit.

collected 2 hour after incubation of TLR ligand with primary GECs were incubated with 1G5 cells. LTR activation similar to mock treated controls was observed in the last 4 of 5 total washes, suggesting that no residual TLR ligands remained in the cell culture supernatants after the last PBS wash (results not shown). These results suggest that specific TLR ligands can directly activate the HIV-LTR in 1G5 T cells, or induce primary GECs to secrete factors that can indirectly activate the HIV-LTR in 1G5 cells.

Copathogens Can Directly and Indirectly Drive the HIV-LTR in 1G5 Cells

Since we observed that TLR ligands could directly drive HIV-LTR activation, we next examined whether herpes simplex viruses or *N. gonorrhoeae* could similarly increase HIV-LTR activation. 1G5 cells exposed directly to HSV-1 KOS HSV-2 333 ($P < .004$) or *N. gonorrhoeae* ($P < .001$) all induced potent HIV-LTR activation in comparison to mock-infected controls (Figure 2a).

To determine whether these common sexually transmitted pathogens could also indirectly induce HIV-LTR activation in the genital tract, 1G5 T cells were incubated with apical and basolateral supernatants from primary GEC cultures grown in transwell inserts and exposed to either HSV-1, HSV-2 or *N. gonorrhoeae*. Apical supernatants collected from HSV-1, HSV-2 or *N. gonorrhoeae*-infected primary GEC cultures activated HIV-LTR in 1G5 T cells (Figure 2b). Of the basolateral supernatants, only those collected from primary GECs exposed to *N. gonorrhoeae* induced HIV-LTR activation (Figure 2b). Basolateral supernatants collected from HSV-1–or HSV-2–infected GECs did not induce HIV-LTR activation. These results suggest that common STI pathogens that coinfect HIV-infected individuals may directly induce HIV-LTR activation in infected T cells, and also cause GECs to secrete immune factors that indirectly drive HIV replication.

Primary GECs Infected With Copathogens Produce Elevated Levels of Proinflammatory Cytokines and Chemokines

GECs can respond to incoming pathogens by producing proinflammatory cytokines and chemokines [29]. These can activate the HIV LTR and promote HIV replication, as well as drive chronic systemic HIV immune activation [18, 19, 30–33]. We therefore measured copathogen-exposed GEC supernatants for 8 different cytokines and chemokines known to be produced by GECs. Apical supernatants from primary GECs exposed to HSV-1, HSV-2 or *N. gonorrhoeae* contained elevated levels of TNF-α, IL-6, IL-8, and MCP-1 compared with unexposed controls (Figure 3a). Basolateral supernatants from

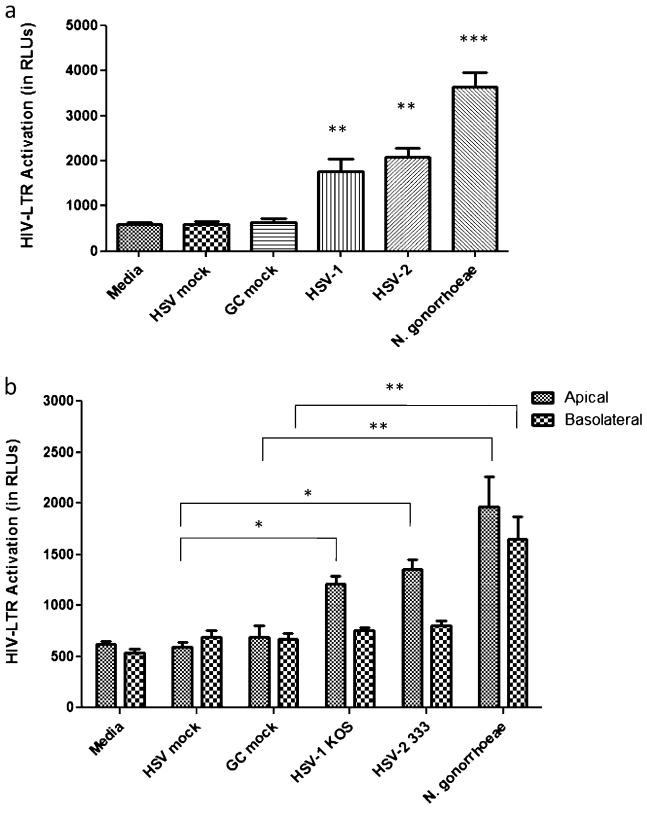

Figure 2. Herpes simplex virus type 1 (HSV-1), HSV-2, and *Neisseria gonorrhoeae* can directly and indirectly increase human immunodeficiency virus (HIV)–long terminal repeat (LTR) activation in 1G5 cells. To assess whether common coinfections of the female genital tract could directly contribute to HIV-LTR activation (*A*), 1×10^6 1G5 cells were infected with 10^4 plaque-forming units (PFUs) of HSV-1 KOS or HSV-2 333 or 10^6 colony-forming units (CFUs) of *N. gonorrhoeae* clinical strain 2071 for 16 h. After this, the cells were lysed and luciferase activity was measured and reported in relative light units (RLUs). Data are mean ± SEM of 3 separate experiments. To assess whether common coinfections of the female genital tract could indirectly contribute to HIV-LTR activation (*B*), primary genital epithelial cell cultures were grown to confluency on transwell inserts and infected with 10^4 PFUs of HSV-2 333 or HSV-1 KOS or 10^6 CFUs of *N. gonorrhoeae* clinical strain 2071 for 2 hours. After this, the inocula were removed and the cells were washed 5 times with 1x phosphate buffered saline and subsequently replenished with fresh media. Twenty-four hours after infection, the apical and basolateral supernatants were collected and either briefly exposed to UV light, or filter sterilized to kill/remove any live virus or bacteria, respectively, remaining in the supernatant. The supernatants were incubated with 1×10^6 1G5 cells for 24 hours, after which the cells were lysed and luciferase activity was determined. Data are mean ± standard error of the mean of 3 separate experiments. An asterisk indicates $P < .05$; a double asterisk indicates $P < .01$, and a triple asterisk indicates $P < .001$.

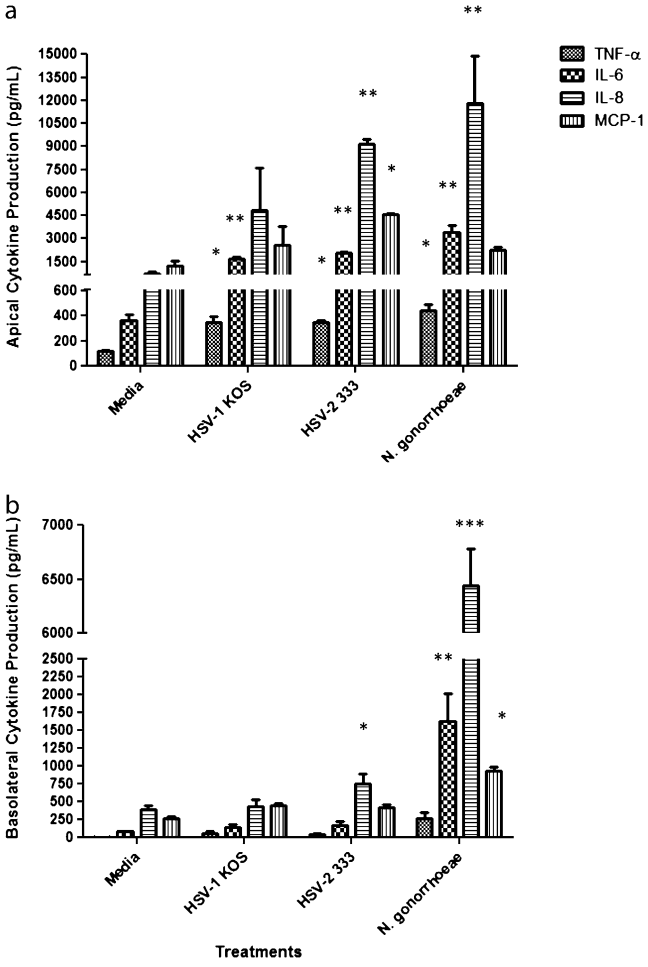

Figure 3. Cytokine and chemokine profiles of apical (*A*) and basolateral (*B*) supernatants from primary endometrial genital epithelial cells (GECs) infected with herpes simplex virus type 1 (HSV-1), HSV-2, or *Neisseria gonorrhoeae*. Confluent primary endometrial GECs were mock infected or infected with 10^4 PFUs of HSV-2 333 or HSV-1 KOS or 1×10^6 colony-forming units of *N. gonorrhoeae* for 2 hours. After this, the inocula were removed and the cells were washed 5 times with 1x phosphate-buffered saline and subsequently replenished with fresh media. Sixteen hours after infection, apical (*A*) and basolateral (*B*) supernatants were collected and assayed for interleukin 1α, interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α, granulocyte-macrophage colony stimulating factor (GM-CSF), regulated on activation normal T cell expressed and secreted (RANTES), and tumor necrosis factor α (TNF-α) using a multianalyte Luminex assay. Data are mean ± standard error of the mean of 3 separate experiments. An asterisk indicates, $P < .05$; a double asterisk indicates $P < .01$, and a triple asterisk indicates $P < .001$.

N. gonorrhoeae exposed primary GECs contained elevated levels of these cytokines as well (Figure 3b). However, HSV-1–and HSV-2–exposed GECs consistently secreted very low levels of most cytokines into the basolateral supernatant, likely explaining why these basolateral supernatants demonstrated a diminished capacity to indirectly drive the HIV-LTR (Figure 2b). No supernatant contained elevated levels of IL-1α, MIP-1α, GM-CSF, or RANTES following infection (results not shown). These results suggest that in response to common coinfecting pathogens, GECs secrete a number of proinflammatory cytokines and chemokines that have previously been implicated in increasing HIV replication.

The Direct and Indirect Activation of HIV-LTR Can Be Abrogated by Curcumin

Since we observed that proinflammatory factors were produced by primary GECs in response to HSV-1, HSV-2 or *N. gonorrhoeae* exposure, we next blocked intracellular inflammatory pathways by treating 1G5 cells with curcumin, a potent broad-spectrum inhibitor of anti-inflammatory pathways [34], prior to incubation with pathogens or GEC supernatants. Pretreatment of 1G5 cells with curcumin completely blocked the ability of HSV-1, HSV-2 and *N. gonorrhoeae* to directly drive the HIV-LTR (Figure 4a). Furthermore, pretreatment of 1G5 cells with curcumin also completely inhibited indirect HIV-LTR activation by apical cell culture supernatants from primary GECs exposed to HSV-1, HSV-2 or *N. gonorrhoeae* (Figure 4b). Curcumin pretreatment did not affect 1G5 cell viability, as measured by trypan blue cellular staining (not shown) indicating that inhibition of HIV-LTR activation was due specifically to the anti-inflammatory effects of curcumin. This suggests that viral and bacterial coinfections of the genital tract directly activate inflammatory pathways in 1G5 T cells, resulting in direct HIV-LTR activation. Furthermore, these pathogens induce primary GECs to secrete immune factors that can trigger inflammatory pathways in HIV-infected T cells, resulting in indirect HIV-LTR activation.

Direct and Indirect HIV-LTR Activation by Coinfecting Pathogens Requires NFκB and AP-1

Curcumin is a broad-spectrum compound known to inhibit growth factors, antiapoptotic mechanisms, protein kinases, and transcription factors, such as nuclear factor (NF)κB and AP-1, both of which modulate cytokine production and have been shown to play important roles in the induction of HIV replication [34–36]. We next determined if blocking these intracellular inflammatory pathways could abrogate HIV-LTR activation in 1G5 cells. 1G5 cells were pretreated with PDTC, an inhibitor of NFκB activation [37], and inhibitors of p38 mitogen-activated protein (MAP) kinase (SB203580) and c-Jun *N*-terminal kinase (JNK) (SP600125), both of which activate AP-1 [37–39]. 1G5 cells pretreated with pathway-specific inhibitors were exposed to HSV-1, HSV-2, *N. gonorrhoeae,* or cell culture supernatants from primary GECs infected with these pathogens. Direct activation of the HIV-LTR by HSV-1, HSV-2, and *N. gonorrhoeae* was significantly decreased in 1G5 T cells pretreated with specific inhibitors of either NFκB or AP-1. The greatest decrease, comparable to uninfected controls in HIV-LTR activity, was observed when 1G5 T cells were pretreated with a combination of both inhibitors. This indicates that direct activation of HIV-LTR in 1G5 T cells required both NFκB and AP-1 (Figure 5).

The NFκB and AP-1 pathways were also found to be the main intracellular pathways involved in indirect HIV-LTR activation by GEC supernatants as the dual blockade of NFκB and AP-1 led to greatest abrogation of indirect HIV-LTR activation in 1G5 cells (Figure 6). The p38 MAP kinase did not seem to play a significant role in this process, as neither its inhibition individually nor in concert with the other inhibitors had a significant effect on HIV-LTR activation in 1G5 cells (Figure 7). These results suggest that both direct and indirect activation of HIV-LTR is mediated primarily by NFκB and JNK-mediated AP-1 signaling pathways in infected T cells.

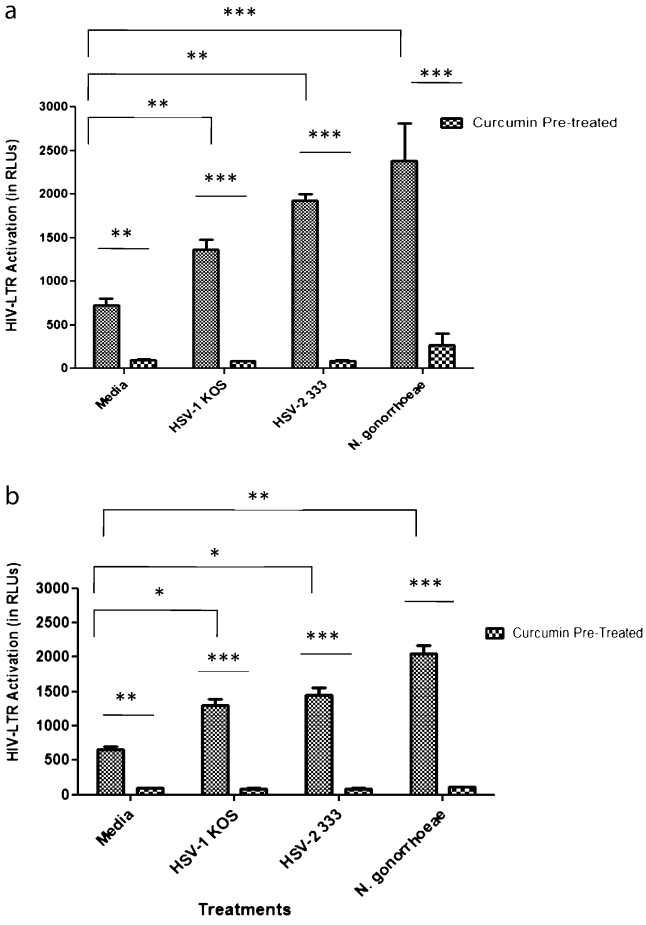

Figure 4. The direct and indirect effect of herpes simplex virus type 1 (HSV-1), HSV-2, and *Neisseria gonorrhoeae* infection on human immunodeficiency virus (HIV)–long terminal repeat (LTR) activation in 1G5 cells is inhibited by curcumin. To measure whether proinflammatory pathways played a role in inducing direct (*A*) or indirect (*B*) HIV-LTR activation, 5 μM of curcumin was added to 1×10^6 1G5 cells for 1 hour. The cells were then washed and either infected with 10^4 plaque-forming units (PFUs) HSV-1 KOS or HSV-2 333 or 10^6 colony-forming units (CFUs) *N. gonorrhoeae* for 16 h (*A*); or they were exposed for 24 hours to apical supernatants collected from primary GECs infected with 10^4 pfus HSV-1 KOS or HSV-2 333 or 10^6 CFUs *N. gonorrhoeae,* as previously described (*B*). Data are mean ± standard error of the mean of 3 separate experiments. An asterisk indicates $P < .05$, a double asterisk indicates $P < .01$, and a triple asterisk indicates $P < .001$; RLU, relative light unit.

DISCUSSION

In this study, we addressed how heterologous coinfection with HSV-1, HSV-2, or *N. gonorrhoeae* can influence HIV replication

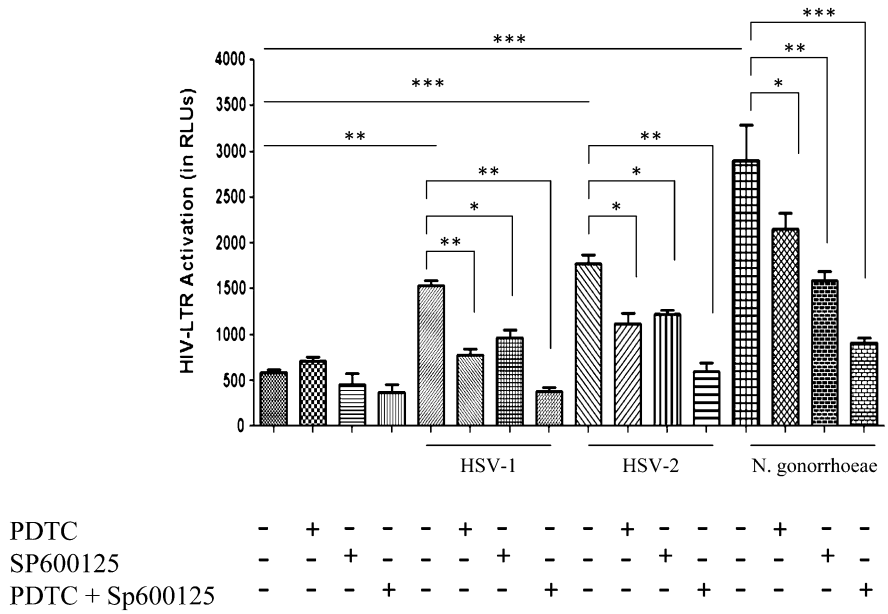

Figure 5. Direct human immunodeficiency virus (HIV)–long terminal repeat (LTR) activation by herpes simplex virus type 1 (HSV-1), HSV-2, and *Neisseria gonorrhoeae* in 1G5 cells is dependent on nuclear factor κB (NFκB) and AP-1. To determine whether direct HIV-LTR activation required NFκB or AP-1, 1G5 cells were pretreated with either 10 μM PDTC or SP600125 for 1 hour. The cells were then washed thoroughly with 1x phosphate buffered saline before being infected with HSV-1, HSV-2, or *N. gonorrhoeae* as previously described. The cells were then lysed and luciferase activity was measured. Data are mean ± standard error of the mean of 3 separate experiments. An asterisk indicates $P < .05$, a double asterisk indicates $P < .01$, and a triple asterisk indicates $P < .001$; RLU, relative light unit.

in infected T cells in the upper female genital tract. We found that TLR ligands, representative of viral (Poly I:C) and bacterial (FimH, flagellin) coinfections, as well as the common sexually transmitted pathogens HSV-1, HSV-2, and *N. gonorrhoeae*, were capable of directly activating the HIV-LTR promoter in stably transfected T cells. Furthermore, these TLR ligands and

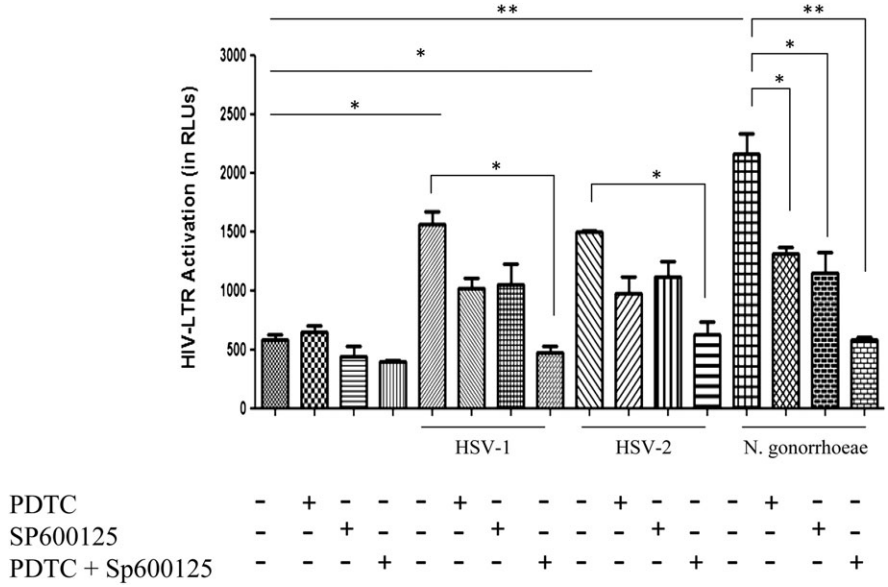

Figure 6. Indirect human immunodeficiency virus (HIV)–long terminal repeat (LTR) activation by herpes simplex virus type 1 (HSV-1), HSV-2, and *Neisseria gonorrhoeae* in 1G5 cells relies on nuclear factor κB (NFκB) and AP-1. To determine whether indirect HIV-LTR activation required NFκB or AP-1, 1G5 cells were pretreated with either 10 μM PDTC or SP600125 for 1 hour. The cells were then washed thoroughly with 1x phosphate-buffered saline before being incubated with supernatants from primary GECs that had been infected with HSV-1, HSV-2, or *N. gonorrhoeae* as previously described. After incubation, the 1G5 cells were lysed and luciferase activity was measured. Data are mean ± standard error of the mean of 3 separate experiments. An asterisk indicates $P < .05$, a double asterisk indicates $P < .01$ and a triple asterisk indicates $P < .001$; RLU, relative light unit.

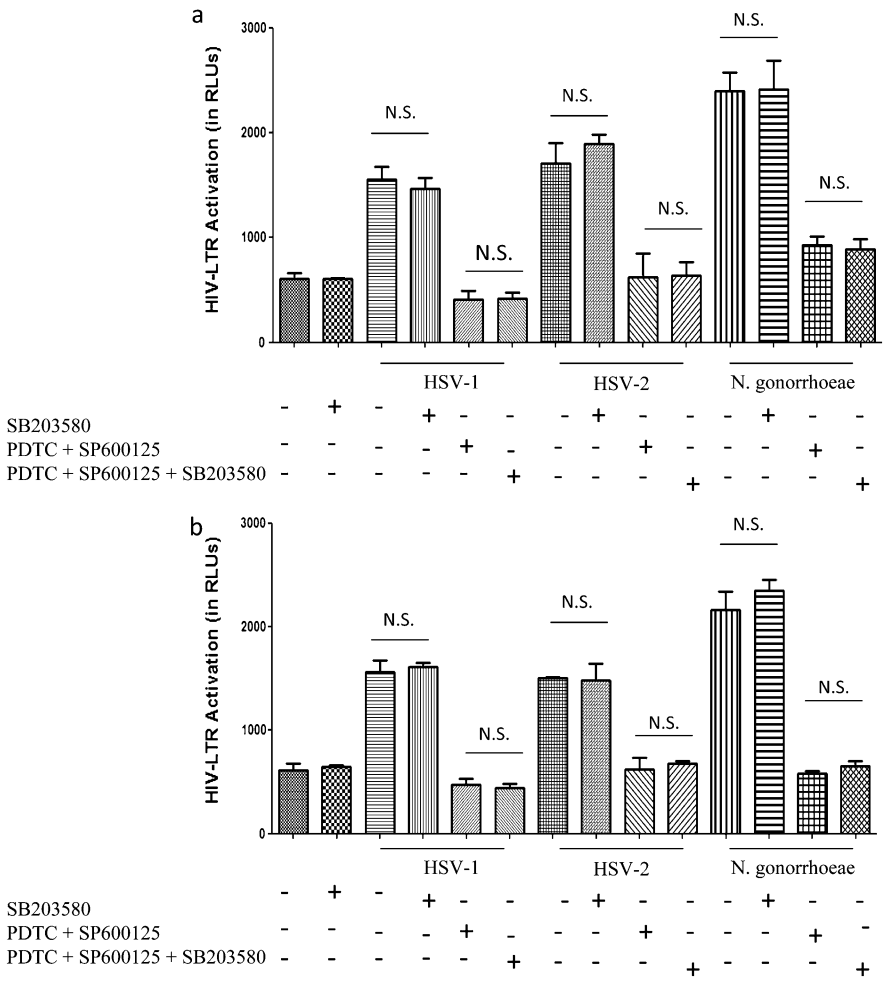

Figure 7. The direct and indirect effect of herpes simplex virus type 1 (HSV-1), HSV-2, and *Neisseria gonorrhoeae* infection on human immunodeficiency virus (HIV)–long terminal repeat (LTR) activation in 1G5 cells does not depend on p38 MAP kinase. To determine whether the AP-1 activating enzyme p38 MAP kinase played an important role in inducing direct (*A*) or indirect (*B*) HIV-LTR activation, 1×10^6 1G5 cells were pretreated singularly with 10 μM SB203580, or with SB203580 in combination with 10 μM of PDTC and SP600125 for 1 hour. The cells were then washed and either infected with 10^4 plaque-forming units HSV-1 KOS or HSV-2 333 or 10^6 colony-forming units (CFUs) *N. gonorrhoeae* for 16 hours (*A*); or they were exposed for 24 h to apical supernatants collected from primary GECs infected with 10^4 PFUs HSV-1 KOS or HSV-2 333 or 10^6 CFUs *N. gonorrhoeae,* as previously described (*B*). Data are mean ± standard error of the mean of 3 separate experiments. An asterisk indicates $P < .05$, a double asterisk indicates $P < .01$, and a triple asterisk indicates $P < .001$; N.S., not significant; RLU, relative light unit.

coinfecting pathogens induced GEC to secrete immune factors that indirectly activated the HIV-LTR promoter. Key factors secreted by GECs that could activate the HIV-LTR included the proinflammatory cytokines TNF-α and IL-6 and chemokines IL-8 and MCP-1. The direct and indirect activation of the HIV-LTR was completely abrogated when anti-inflammatory pathways were blocked. The direct and indirect activation of the HIV-LTR was mediated by transcription factors NFκB and AP-1.

These results support previous studies that have suggested that common sexually transmitted pathogens, such as HSV-1, HSV-2, and *N. gonorrhoeae,* are all capable of directly inducing HIV replication [8, 28, 40–42]. In addition, the present study shows that recognition of pathogens by TLRs on infected T cells could directly activate proinflammatory signaling pathways, thereby activating HIV-LTR. More importantly, the present study provides new insight into the effect of a proinflammatory milieu in the upper female genital tract on HIV replication. Bacterial TLR ligands, FimH and flagellin, as well as HSV-1, HSV-2, and *N. gonorrhoeae,* were all seen to induce GECs to secrete proinflammatory cytokines and chemokines that indirectly contributed to increasing HIV replication in transfected T cells. These results suggest that direct contact between a pathogen and a T cell harboring proviral HIV DNA may not be necessary to induce HIV replication in coinfected individuals. STIs could enhance HIV replication by inducing production of proinflammatory cytokines and chemokines from GECs in the genital tract, and this may be sufficient to enhance HIV

replication. These results are supported by clinical observations that among HSV-2/HIV coinfected individuals, increased HIV shedding was most dramatic among women with elevated chemokines and proinflammatory cytokine levels in cervico-vaginal lavage fluid [43].

Another novel finding in the present study was that bacterial and viral copathogens in the genital tract may induce distinct responses from GECs, resulting in differential potentiation of HIV-LTR activation. In response to HSV-1 and HSV-2 infection, primary GECs secreted proinflammatory factors that could indirectly drive HIV-LTR activation only onto the apical or luminal side. Conversely, bacterial TLR ligands, as well as *N. gonorrhoeae*, successfully activated the HIV-LTR with supernatants from both the apical and basolateral sides. Since HIV-infected T cells would predominantly be present in the subepithelial lamina propria of the genital mucosa, bacterial infection may be more likely to induce HIV replication indirectly. The apical proinflammatory factors secreted by GECs in response to HSV infection may act in an autocrine manner to upregulate HIV-LTR in GECs. Although controversial, GECs have been shown to be infected by HIV [44–46], and it may be possible that the apical factors induced by HSV-1 and HSV-2 may facilitate HIV replication in these cells. Ongoing studies in our laboratory are examining this possibility.

In the current study, we were able to identify the intracellular pathways involved in direct and indirect induction of HIV-LTR in transfected T cells. Curcumin, a broad-spectrum inhibitor, could abrogate both direct and indirect LTR activation. Unlike "smart drugs," which inhibit 1 factor alone, curcumin has been referred to as a "dirty drug," because it has been shown to suppress multiple signaling pathways [34]. By using specific inhibitors of NFκB and AP-1, transcription factors that have been implicated in HIV transcription [36], we were able to show complete abrogation of LTR activation. In contrast, inhibition of the AP-1 activating MAP kinase p38 did not affect either direct or indirect HIV-LTR activation, suggesting that JNK-mediated AP-1 and NFκB pathways regulate HIV-LTR activation in T cells.

In conclusion this study reveals for, to our knowledge, the first time the cellular mechanism that may drive HIV replication in the presence of viral or bacterial coinfections. These copathogens could directly activate HIV replication in infected T cells in the genital tract. More importantly, even in the absence of any direct contact between pathogens and infected cells, the proinflammatory factors secreted by GECs may be sufficient to activate HIV-LTR. These results indicate that inhibition of inflammatory pathways may also be effective in reducing HIV replication in coinfected individuals in addition to antimicrobial treatments.

Funding

This work was supported by research grants from Ontario HIV Treatment Network (OHTN) and the Canadian Foundation of AIDS Research (CANFAR) and CIHR Emerging Team Grant HET85518. C. K. is the recipient of an OHTN Scholarship Award and a New Investigator's Award from CIHR. V. H. F. is the recipient of an OHTN Studentship Award.

Acknowledgments

The authors thank the Pre-Op Clinic Staff for their cooperation in obtaining informed consents and the Clinical Pathology Staff at McMaster University Medical Centre for their assistance in providing genital tract tissues. We thank the women who donated their tissues for this study.

References

1. Kaul R, Pettengell C, Sheth PM, et al. The genital tract immune milieu: an important determinant of HIV susceptibility and secondary transmission. J Reprod Immunol **2008**; 77:32–40.
2. Cohen MS. Sexually transmitted diseases enhance HIV transmission: no longer a hypothesis. Lancet **1998**; 351(suppl 3):5–7.
3. Blanchard A, Montagnier L, Gougeon ML. Influence of microbial infections on the progression of HIV disease. Trends Microbiol **1997**; 5:326–31.
4. UNAIDS. Report on the global AIDS epidemic 2010. 2011. http://www.unaids.org/globalreport/Global_report.htm. Accessed 4 May 2011.
5. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. Nat Rev Immunol **2008**; 8:737–44.
6. Page KR, Moore RD, Wilgus B, Gindi R, Erbelding EJ. *Neisseria gonorrhoeae* and *Chlamydia trachomatis* among human immunodeficiency virus-infected women. Sex Transm Dis **2008**; 35:859–61.
7. Zhang J, Li G, Bafica A, et al. *Neisseria gonorrhoeae* enhances infection of dendritic cells by HIV type 1. J Immunol **2005**; 174:7995–8002.
8. Ding J, Rapista A, Teleshova N, et al. *Neisseria gonorrhoeae* enhances HIV-1 infection of primary resting $CD4^+$ T cells through TLR2 activation. J Immunol **2010**; 184:2814–24.
9. Ghys PD, Fransen K, Diallo MO, et al. The associations between cervicovaginal HIV shedding, sexually transmitted diseases and immunosuppression in female sex workers in Abidjan, Cote d'Ivoire. AIDS **1997**; 11:F85–93.
10. Centers for Disease Control. Sexually Transmitted Diseases Treatment Guidelines. 2010. http://www.cdc.gov/std/treatment/2010/. Accessed 16 January 2011.
11. Gupta R, Warren T, Wald A. Genital herpes. Lancet **2007**; 370:2127–37.
12. Coyle PV, O'Neill HJ, Wyatt DE, McCaughey C, Quah S, McBride MO. Emergence of herpes simplex type 1 as the main cause of recurrent genital ulcerative disease in women in Northern Ireland. J Clin Virol **2003**; 27:22–9.
13. Roberts CM, Pfister JR, Spear SJ. Increasing proportion of herpes simplex virus type 1 as a cause of genital herpes infection in college students. Sex Transm Dis **2003**; 30:797–800.
14. McClelland RS, Wang CC, Overbaugh J, et al. Association between cervical shedding of herpes simplex virus and HIV-1. AIDS **2002**; 16:2425–30.
15. Schacker T, Zeh J, Hu HL, Hill E, Corey L. Frequency of symptomatic and asymptomatic herpes simplex virus type 2 reactivations among human immunodeficiency virus-infected men. J Infect Dis **1998**; 178:1616–22.
16. Lingappa JR, Baeten JM, Wald A, et al. Daily acyclovir for HIV-1 disease progression in people dually infected with HIV-1 and herpes simplex virus type 2: a randomised placebo-controlled trial. Lancet **2010**; 375:824–33.
17. Royce RA, Sena A, Cates W Jr, Cohen MS. Sexual transmission of HIV. N Engl J Med **1997**; 336:1072–8.
18. Poli G, Bressler P, Kinter A, et al. Interleukin 6 induces human immunodeficiency virus expression in infected monocytic cells alone and in synergy with tumor necrosis factor alpha by transcriptional and post-transcriptional mechanisms. J Exp Med **1990**; 172:151–8.
19. Poli G, Kinter A, Justement JS, et al. Tumor necrosis factor alpha functions in an autocrine manner in the induction of human immunodeficiency virus expression. Proc Natl Acad Sci U S A **1990**; 87:782–5.

20. Nazli A, Chan O, Dobson-Belaire WN, et al. Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. PLoS Pathog **2010**; 6:e1000852.
21. Gillgrass AE, Tang VA, Towarnicki KM, Rosenthal KL, Kaushic C. Protection against genital herpes infection in mice immunized under different hormonal conditions correlates with induction of vagina-associated lymphoid tissue. J Virol **2005**; 79:3117–26.
22. Dobson-Belaire WN, Rebbapragada A, Malott RJ, et al. *Neisseria gonorrhoeae* effectively blocks HIV-1 replication by eliciting a potent TLR9-dependent interferon-alpha response from plasmacytoid dendritic cells. Cell Microbiol **2010**; 12:1703–17.
23. Nazli A, Yao XD, Smieja M, Rosenthal KL, Ashkar AA, Kaushic C. Differential induction of innate anti-viral responses by TLR ligands against herpes simplex virus, type 2, infection in primary genital epithelium of women. Antiviral Res **2009**; 81:103–12.
24. MacDonald EM, Savoy A, Gillgrass A, 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**; 77:1049–59.
25. Ashkar AA, Mossman KL, Coombes BK, Gyles CL, Mackenzie R. FimH adhesin of type 1 fimbriae is a potent inducer of innate antimicrobial responses which requires TLR4 and type 1 interferon signalling. PLoS Pathog **2008**; 4:e1000233.
26. Mossman KL, Mian MF, Lauzon NM, et al. Cutting edge: FimH adhesin of type 1 fimbriae is a novel TLR4 ligand. J Immunol **2008**; 181:6702–6.
27. Fernandez S, Gillgrass A, Kaushic C. Differential responses of murine vaginal and uterine epithelial cells prior to and following herpes simplex virus type 2 (HSV-2) infection. Am J Reprod Immunol **2007**; 57:367–77.
28. Chen A, Boulton IC, Pongoski J, Cochrane A, Gray-Owen SD. Induction of HIV-1 long terminal repeat-mediated transcription by *Neisseria gonorrhoeae*. AIDS **2003**; 17:625–8.
29. Li H, Li X, Wei Y, Tan Y, Liu X, Wu X. HSV-2 induces TLRs and NF-kappaB-dependent cytokines in cervical epithelial cells. Biochem Biophys Res Commun **2009**; 379:686–90.
30. Mellors JW, Griffith BP, Ortiz MA, Landry ML, Ryan JL. Tumor necrosis factor-alpha/cachectin enhances human immunodeficiency virus type 1 replication in primary macrophages. J Infect Dis **1991**; 163:78–82.
31. Fahey JL. Cytokines, plasma immune activation markers, and clinically relevant surrogate markers in human immunodeficiency virus infection. Clin Diagn Lab Immunol **1998**; 5:597–603.
32. Aziz N, Nishanian P, Fahey JL. Levels of cytokines and immune activation markers in plasma in human immunodeficiency virus infection: quality control procedures. Clin Diagn Lab Immunol **1998**; 5:755–61.
33. Roberts L, Passmore JA, Williamson C, et al. Plasma cytokine levels during acute HIV-1 infection predict HIV disease progression. AIDS **2010**; 24:819–31.
34. Kunnumakkara AB, Anand P, Aggarwal BB. Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. Cancer Lett **2008**; 269:199–225.
35. Duh EJ, Maury WJ, Folks TM, Fauci AS, Rabson AB. Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. Proc Natl Acad Sci U S A **1989**; 86:5974–8.
36. Pereira LA, Bentley K, Peeters A, Churchill MJ, Deacon NJ. A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. Nucleic Acids Res **2000**; 28:663–8.
37. Schreck R, Meier B, Mannel DN, Droge W, Baeuerle PA. Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. J Exp Med **1992**; 175:1181–94.
38. Jarnicki AG, Conroy H, Brereton C, et al. Attenuating regulatory T cell induction by TLR agonists through inhibition of p38 MAPK signaling in dendritic cells enhances their efficacy as vaccine adjuvants and cancer immunotherapeutics. J Immunol **2008**; 180:3797–806.
39. Bennett BL, Sasaki DT, Murray BW, et al. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A **2001**; 98:13681–6.
40. Schafer SL, Hiscott J, Pitha PM. Differential regulation of the HIV-1 LTR by specific NF-kappa B subunits in HSV-1-infected cells. Virology **1996**; 224:214–23.
41. Margolis DM, Rabson AB, Straus SE, Ostrove JM. Transactivation of the HIV-1 LTR by HSV-1 immediate-early genes. Virology **1992**; 186:788–91.
42. Golden MP, Kim S, Hammer SM, et al. Activation of human immunodeficiency virus by herpes simplex virus. J Infect Dis **1992**; 166:494–9.
43. Rebbapragada A, Wachihi C, Pettengell C, et al. Negative mucosal synergy between herpes simplex type 2 and HIV in the female genital tract. AIDS **2007**; 21:589–98.
44. Howell AL, Edkins RD, Rier SE, et al. Human immunodeficiency virus type 1 infection of cells and tissues from the upper and lower human female reproductive tract. J Virol **1997**; 71:3498–506.
45. Dezzutti CS, Guenthner PC, Cummins JE Jr., et al. Cervical and prostate primary epithelial cells are not productively infected but sequester human immunodeficiency virus type 1. J Infect Dis **2001**; 183:1204–13.
46. Wu Z, Chen Z, Phillips DM. Human genital epithelial cells capture cell-free human immunodeficiency virus type 1 and transmit the virus to $CD4^+$ cells: implications for mechanisms of sexual transmission. J Infect Dis **2003**; 188:1473–82.