HIV-1 gp120 Induces TLR2- and TLR4-Mediated Innate Immune Activation in Human Female Genital Epithelium


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HIV-1 gp120 Induces TLR2- and TLR4-Mediated Innate Immune Activation in Human Female Genital Epithelium


Although women constitute half of all HIV-1–infected people worldwide (UNAIDS World AIDS Day Report, 2011), the earliest events in the female reproductive tract (FRT) during heterosexual HIV-1 transmission are poorly understood. Recently, we demonstrated that HIV-1 could directly impair the mucosal epithelial barrier in the FRT. This suggested that the HIV-1 envelope glycoprotein gp120 was being recognized by a membrane receptor on genital epithelial cells, leading to innate immune activation. In this study, we report that pattern-recognition receptors TLR2 and -4 bind to HIV-1 gp120 and trigger proinflammatory cytokine production via activation of NF-κB. The gp120–TLR interaction also required the presence of heparan sulfate (HS). Bead-binding assays showed that gp120 can bind to HS, TLR2, and TLR4, and studies in transfected HEK293 cells demonstrated that HS and TLR2 and -4 were necessary to mediate downstream signaling. Exposure to seminal plasma from HIV-1–infected and uninfected men with gp120 added to it induced a significant proinflammatory cytokine response from genital epithelial cells and disruption of tight junctions, indicating a role for gp120 in mucosal barrier disruption during HIV-1 heterosexual transmission. These studies provide, for the first time to our knowledge, a possible mechanism by which HIV-1 gp120 could directly initiate innate immune activation in the FRT during heterosexual transmission. The Journal of Immunology, 2013, 191: 4246–4258.
microbial translocation across the intestinal and genital mucosa and is concomitant with chronic systemic inflammation, as detected by measurable levels of serum LPS and soluble CD14 (8, 9). Presence of these factors in the blood has been directly correlated with disease progression (10, 11).

The exact mechanism by which HIV participates in the generation of immune activation is not known, but our previous studies implicate the viral glycoprotein gp120 as a primary mediator of this response. Cells infected with HIV-1 in vitro spontaneously shed the gp120 envelope protein in quantities greater than other viral proteins (12, 13), and detection of plasma gp120 levels have been associated with higher plasma concentrations of IL-6 and TNF-α in acutely infected (AI) individuals (14). Overall, studies suggest a paradoxical role, with some evidence for gp120-mediated suppression of T cell and DC function (15–18), whereas others show activation of CD4+ T lymphocytes and myeloid cells, in the absence of direct infection (19, 20).

In the current study, we report for the first time, to our knowledge, the mechanism by which HIV-1 gp120 activates proinflammatory pathways in GECs. Our results show that gp120 signals through TLR2 and -4 in the presence of heparan sulfate (HS). We propose that gp120 in seminal plasma (SP) can initiate TLR-mediated activation of proinflammatory pathways that could lead to the disruption of the mucosal barrier and the initiation of immune activation by HIV-1.

Materials and Methods

Patient participation

Endometrial and endocervical tissues were obtained from women aged 30–59 y (mean age 42.9 ± 7.2 y) undergoing hysterectomies for benign gynecological reasons at Hamilton Health Sciences Hospital. The most common reasons for surgery were uterine fibroids and menorrhagia (heavy bleeding). Informed written consent was obtained in accordance with the approval of the Hamilton Health Sciences Research Ethics Board. HIV-infected, antiretroviral therapy–naïve men were recruited through the Canadian Immunodeficiency Research Collaborative; all were men who have sex with men. Uninfected SP was obtained from either heterosexual male or recombinant gp120 (0.1 μg/ml) for various time points. Mock infection controls included exposure to the same volume of media without HIV-1.

To explore the role of cell-surface receptors on various aspects of HIV/GE C interactions, epithelial monolayers were treated with neutralizing Abs against TLR2 (eBioscience; clone TL2.1), TLR4 (eBioscience; clone HTA125), TLR5 (Invivogen; polyclonal rat anti-human) (all at 10 μg/ml), CD4 (DakoCytomation; clone MT310, 20 μg/ml) (25), CCR5 (BD Biosciences; clone 2D7/CCCR5, 20 μg/ml) (26), CXCRI (BD Biosciences; clone 12G5, 20 μg/ml) (27), or their respective isotype controls at similar concentrations during the course of viral exposure. In some experiments, digested HS and chondroitin sulfate (CS), and phospholipase, respectively. Exogenous HS (Sigma-Aldrich; 40 μg/ml) was added in some experiments to replace cell-surface receptors. The concentrations of these Abs or inhibitors were based on previous optimization experiments.

To block the effect of gp120, a specific neutralizing Ab (Polymun Diagnostic, Klosterneuburg, Austria; clone 2G12, 25 μg/ml) was added to primary GEC cultures for the duration of viral exposure. As positive controls for TLR2, TLR4, and TLR5 signaling pathways, primary GECs were exposed to Pam3CSK4 (InvivoGen, San Diego, CA), FimH (28), and flagellin from Salmonella typhimurium (Alpha Diagnostic, San Antonio, TX), respectively, at a concentration of 10 μg/ml.

Immunofluorescence staining

GECs were exposed to HIV-1, gp120, or polyinosinic-polycytidylic acid [poly (I:C)] (Sigma-Aldrich; 25 μg/ml; positive control) (28) and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), Primary antibody anti–NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti–anti-human zona occludens-1 (ZO-1, Life Technologies, Burlington, ON, Canada) Ab was incubated for 1 h at 37˚C followed by secondary Alexa Fluor 488–conjugated (Life Technologies) Ab for 1 h. Cells were counterstained with propidium iodide (nuclear stain; Life Technologies). Membranes were excised from inserts and mounted on slides prior to being imaged on an inverted confocal laser-scanning LSM 510 microscope (Carl Zeiss Canada, Toronto, ON, Canada) using standard operating conditions. Images were presented as en face or as a composite Z-stack reconstruction, which shows the monolayer in transverse profile. Images were analyzed using Image J software (National Institutes of Health) for measuring levels of nuclear colocalization of NF-κB.

Cytokine analysis

GEC apical and basolateral supernatants were analyzed for cytokines and chemokines at several time points post–HIV-1 ADA, UV-inactivated HIV-1, HIV env+, or recombinant gp120 exposure, using Luminex multianalyte technology (Luminex, Austin, TX), as previously described (7). Multiplex bead-based sandwich immunoassay kits (Millipore) were used to measure levels of IL-1β, IL-6, IL-8, MCP-1, and TNF-α. In some experiments, TNF-α and IL-8 in cell-culture supernatants were measured by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Baseline cytokine levels were found to vary between different tissue samples, but in all tissues, significant induction of cytokines was observed reproducibly after treatment with HIV-1 or recombinant gp120. To confirm...
that proinflammatory cytokine induction in primary GECs was primarily mediated by NF-κB, cells were pretreated with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich; 10 μM) for 1 h prior to exposure with either gp120 or HIV-1. Cell-culture supernatants were subsequently collected and measured by bead-based immunoassay.

**Blue dextran leakage assay**

Blue dextran dye (Sigma Aldrich) was dissolved in primary medium to a working concentration of 2.3 mg/ml (7) and added to the apical surface of confluent epithelial cell monolayers grown to confluence on transwell inserts. At 24 h post–gp120 exposure, 100 μl basalateral supernatant was collected, loaded into 96-well plates, and measured in a Tecan Safire microplate reader (Tecan, Männedorf, Switzerland) at 610 nm. Blue dextran leakage into the basolateral compartment was expressed as a percent of the dye added to the apical surface.

**TLR, gp120, and HS bead-binding assay**

Polystyrene microspheres (4.5 μm; Polysciences, Warrington, PA) were washed in borate buffer (0.1 M) according to the manufacturer’s instructions (Tech note 238E, Polysciences). Washed microbeads (0.25 mg/ml) were coated at room temperature with 1 μg/ml recombinant gp120, human TLR2, human TLR4, 50 μg/ml HS, or 10 μg/ml BSA in borate buffer (pH 8.5) overnight with vigorous shaking. Following a buffer wash, two 30-min incubations were performed with 10 μg/ml BSA in borate buffer and 10 μg/ml BSA in PBS to block nonspecific binding on all beads. The coated beads were resuspended in FACS buffer (2 mg/ml BSA in PBS) at 0.5 × 10⁶ beads/ml and stored at -4°C. For binding assays, coated beads were incubated with gp120 (0.5 μg/ml) or TLR4 (0.5 μg/ml) for 1 h at room temperature in FACS buffer, washed, and then incubated for 30 min with biotinylated anti-gp120 or anti-TLR2 or -4Ab followed by a 20-min staining with streptavidin-PE-Cy7. Beads were subsequently analyzed by using a BD LSRII Flow cytometer (BD Biosciences). Single beads were selected for analysis on the scatter plots, and mean fluorescence intensity (MFI) of gp120 or TLR4 Ab staining on coated beads was compared with BSA controls to analyze gp120 binding to TLR2, TLR4, or HS.

**Plasmids and HEK293 transfection assays**

Expression vectors for human TLR2, TLR4, CD14, MD2, β-galactosidase (β-gal), and NF-κB–luc inducible reporter plasmid were kindly provided by Dr. Cynthia Leifer (Cornell University). All plasmids were amplified and purified using Endo-free Midi Prep columns (Qiagen, Toronto, ON, Canada). Transfections for luciferase assays were carried out using the HEK293 cell line. Subconfluent HEK293 cells were transfected with 100 ng pSV β-galactosidase expression plasmid (Stratagene, La Jolla, CA) and 1 μg NF-κB–luc reporter plasmid (firefly lucerase, experimental reporter), 30 ng pGPG-hTLR4 or pUNO-hTLR4 with or without 30 ng CD14, and pUNO-hMD2 expression plasmids and pBabe (empty vector) for a total of 1 μg DNA per well. Transfections were completed using Gene Juice transfection reagent (EMD Millipore). At 24 h posttransfection, cells were treated with Pam3CSK4 (TLR2 ligand; 100 μg/ml), Pam3CSK4 plus LPS (TLR4 ligand; 100 ng/ml) or gp120 (0.1, 1.0, and 10 μg/ml). Alternatively, transiently transfected HEK 293 cells were treated with or without heparinase III (6 U/ml) and/or exogenous HS (40 μg/ml) for 1 h prior to gp120 stimulation (0.1 μg/ml). At 48 h posttreatment, the cells were harvested, lysed, and luciferase activity was measured using a Stratagene luciferase assay kit (Agilent Technologies, Mississauga, ON, Canada) as per the manufacturer’s instructions. Activity of NF-κB–luc was also measured by reporter assay (Luciferase Detection kit II; Clontech Laboratories, Mountain View, CA) according to the manufacturer’s instructions. The fold increase in NF-κB luciferase activity was normalized with β-gal expression as an internal control of transfection.

**SP and primary GEC exposure**

Confluent monolayers of primary GECs were exposed for 24 h to uninfected SP spiked with 0.1, 0.2, or 0.3 μg/ml recombinant gp120 (Immunodiagnostics, Woburn, MA) or uninfected/infected SP alone. Recombinant gp120 (0.1 μg/ml) was used as a positive control for comparison. TNF-α production was measured in apical supernatants using TNF-α ELISA as per the manufacturer’s instructions (R&D Systems), and cells were fixed for immunofluorescent staining of ZO-1 tight junctions. Primary GECs were also exposed for 24 h to pooled, diluted SP samples from three AI subjects (acute SP), acute SP combined with recombinant gp120 (0.1 μg/ml), acute SP combined with recombinant gp120 (0.1 μg/ml), and anti-gp120 Ab (35 μg/ml; Polymun Scientific) or acute SP with recombinant gp120 (0.1 μg/ml) and isotype control Ab (35 μg/ml; Southern Biotechnologies Associates, Birmingham, AL). Mock infection or recombinant gp120 (0.1 μg/ml) were used as controls.

**Statistical analysis**

GraphPad Prism Version 4 (GraphPad Software, San Diego, CA) was used to compare three or more means by one-way or two-way ANOVA, depending on the experimental conditions. When an overall statistically significant difference was measured (p < 0.05), a Bonferroni posttest was performed to adjust the p value for multiple comparisons. The p values and the respective comparison for which they were calculated are indicated in the figure legends.

**Results**

**Kinetics and specificity of HIV-1 gp120 for induction of proinflammatory cytokines by GECs**

Previously, we demonstrated that HIV-1 could directly disrupt epithelial barrier function in cultured intestinal or primary endometrial GECs following exposure to HIV-1 or gp120 (7), but we did not measure whether similar events occurred in the endocervix. To confirm that the phenomenon of HIV-1 and gp120-mediated barrier disruption was also applicable to primary endocervical GECs, we exposed endocervical monolayers to gp120 or UV-inactivated HIV-1 ADA and measured a significant decrease in TER (results not shown), upregulation of TNF-α (Fig. 1A), and disruption of epithelial barrier integrity (Fig. 1B), relative to unexposed controls. This barrier impairment and upregulation of TNF-α was analogous to what was previously measured in endometrial GECs following exposure to HIV-1 (7). For further experiments examining the mechanism of barrier function, endometrial tissues were used because they were more abundantly available; however, endocervical epithelial cell cultures were used to confirm key findings.

In our previous study examining the barrier impairing function of HIV-1, we did not directly measure whether GEC proinflammatory cytokine induction differed with respect to whether the cells were exposed to HIV-1 or gp120. Thus, confluent, polarized monolayers of GECs were exposed to HIV-1 or recombinant gp120 protein at previously standardized doses (7), and apical and basolateral supernatants were analyzed for the proinflammatory cytokines TNF-α, IL-6, and IL-1α and chemokines IL-8 and MCP-1. Significantly enhanced production was observed for all cytokines and chemokines; in particular, TNF-α and IL-8 were upregulated in response to both HIV-1 (p < 0.05) and gp120 (p < 0.01), as early as 1 h postexposure (Fig. 2A), compared with mock-infected controls.

To determine whether HIV-1 gp120 alone was sufficient for facilitating this effect, we compared GEC responses to HIV-1, gp120, UV-inactivated HIV-1 and an env-deleted mutant, which lacks the HIV-1 viral envelope precursor gp160 (30). HIV-1 and UV-inactivated HIV-1 enhanced the levels of cytokine production by GECs, comparable with levels induced by recombinant gp120 protein exposure. In contrast, the env-deleted HIV-1 mutant failed to induce proinflammatory cytokines in GECs; cytokine levels in this treatment group were comparable to mock exposure (Fig. 2B).

To confirm the specificity of gp120 in GEC proinflammatory cytokine induction, viral preparations as well as recombinant
gp120 were incubated with anti-gp120–neutralizing Ab prior to exposure to GECs. gp120 neutralization reduced proinflammatory cytokine induction to baseline levels (Fig. 2C). Altogether, these results indicate that HIV-1 gp120 can directly induce a proinflammatory cytokine response in GECs.

**Induction of proinflammatory cytokines by HIV-1 gp120 is mediated through NF-κB activation**

Because proinflammatory cytokines can be induced in GECs by different intracellular pathways, we examined the involvement of the transcription factor NF-κB, which is strongly associated with the induction of downstream proinflammatory responses (31), following HIV-1 or gp120 exposure. GEC monolayers were exposed to HIV-1 and gp120, and the p65 subunit of NF-κB was detected. Nuclear translocation of NF-κB was observed following 2 h of exposure to HIV-1 or gp120 (Fig. 3A). Peak translocation of NF-κB was observed between 30 min to 2 h postexposure with HIV-1 or gp120 (Fig. 3B). Treatment with PDTC, an NF-κB-specific inhibitor, significantly inhibited TNF-α and IL-8 upregulation (Fig. 3C, 3D), indicating that NF-κB was necessary for the induction of proinflammatory cytokines by gp120 and HIV-1. Because the induction of cytokines and intracellular signaling pathways were identical for HIV-1 and gp120, we focused on the mechanism of gp120 for the rest of the study.

**gp120-mediated cytokine induction and barrier disruption occurs via TLR2 and TLR4 signaling pathways**

We next sought to determine whether TLRs played a role in gp120-mediated cytokine induction and epithelial barrier disruption, as NF-κB induction has been associated with multiple TLR signaling pathways. We examined three distinct cell-surface TLRs—TLR2, TLR4, and TLR5—because these three TLRs are primarily responsible for recognizing glycoproteins at the surface of cells and inducing intracellular signaling (32). TLR1 and -6 are also associated with cell-surface protein recognition, but act in association with TLR2 and were therefore not included in our assessment. We first measured whether TLR2, -4, or -5 were associated with gp120-mediated induction of proinflammatory cytokines. GECs were treated with neutralizing Abs against TLR2, TLR4, and TLR5 and exposed to gp120. In the presence of either TLR2 or TLR4 Ab, induction of TNF-α and IL-8 by gp120 was partially, but significantly, blocked (Fig. 4A, 4B). However, treatment with a combination of TLR2- and TLR4-neutralizing Abs reduced cytokine production to baseline. In contrast, treatment of GECs with isotype control or TLR5 neutralizing Ab did not have any effect on gp120-mediated cytokine production. The neutralizing capacity of the TLR5 Ab was confirmed by exposing primary GEC monolayers to TLR5 ligand flagellin in the presence or absence of the neutralizing Ab or isotype control (Supplemental Fig. 1). Pam3CSK4, a synthetic triacylated lipopeptide, known to act through TLR2 and TLR5 (fimbriate protein), a TLR ligand that is known to induce this pathway in GECs, were used as controls to determine the specificity of the TLR2 and -4 Abs (24, 28).

To confirm that cytokine production induced by gp120 activation of TLR2 and TLR4 was associated with barrier disruption, we directly examined barrier disruption in the presence of the TLR-neutralizing Abs (Fig. 4C–E). Monolayers treated with gp120 following preincubation with Abs against TLR2, TLR4, or both receptors did not show a significant decrease in TER (Fig. 4C), suggesting that disruption of barrier integrity involves TLR2 and -4 signaling pathways. In contrast, TLR5 or isotype control Ab did not prevent a gp120-mediated drop in TER. Immunofluorescence staining of ZO-1, an epithelial cell tight junction barrier protein that is disrupted by HIV-1 gp120 (7), was in concurrence with the TER results (Fig. 4E). Measurement of dextran blue dye leakage across the epithelial monolayer of gp120 exposed GECs, in the presence or absence of TLR Abs, also confirmed that epithelial cell permeability was completely abrogated in the presence of both TLR2- and TLR4-neutralizing Abs (Fig. 4D). In contrast, TLR5 Ab did not block dye leakage in the presence of gp120, confirming that gp120 activated proinflammatory cytokines via TLR2 and TLR4, but not TLR5.

**NF-κB translocation mediated by gp120 is blocked in the presence of TLR2 and TLR4 Abs**

We next sought to determine if neutralizing Abs to TLR2 and TLR4 abrogated gp120-mediated activation and nuclear translocation of NF-κB. Incubation of GEC monolayers with Abs against TLR2, TLR4, or both receptors did not show a significant decrease in TER (Fig. 4C), suggesting that disruption of barrier integrity involves TLR2 and -4 signaling pathways. In contrast, TLR5 or isotype control Ab did not prevent a gp120-mediated drop in TER. Immunofluorescence staining of ZO-1, an epithelial cell tight junction barrier protein that is disrupted by HIV-1 gp120 (7), was in concurrence with the TER results (Fig. 4E). Measurement of dextran blue dye leakage across the epithelial monolayer of gp120 exposed GECs, in the presence or absence of TLR Abs, also confirmed that epithelial cell permeability was completely abrogated in the presence of both TLR2- and TLR4-neutralizing Abs (Fig. 4D). In contrast, TLR5 Ab did not block dye leakage in the presence of gp120, confirming that gp120 activated proinflammatory cytokines via TLR2 and TLR4, but not TLR5.

**HS is required for gp120-mediated proinflammatory cytokine induction and barrier disruption**

Next, we determined whether signaling through any of the known canonical HIV-1 receptors contributed to the disruption of the GEC barrier or mediated cytokine induction. Intracellular signaling pathways, in addition to TLR2 and TLR4. Pretreatment of GEC monolayers with neutralizing Abs against CD4, CXCR4, or CCR5 did not have any effect on gp120-mediated decrease in TER, disruption of ZO-1 staining, or induction of proinflammatory cytokines (Fig. 6A–C), suggesting that canonical HIV-1 receptors were not involved in these GEC signaling pathways.

Cellular proteoglycans, such as syndecans, have also been previously shown to be important for HIV-1 gp120 attachment, including on epithelial cells (33–36). We therefore examined if HS or CS moieties played a role in gp120-mediated signaling in GECs. Heparinase III, chondroitinase ABC, and phospholipase C were used to remove HS, CS, or phospholipids (as control), re-
spectively, from the cell surface prior to gp120 treatment. Removal of HS resulted in complete blockade of gp120-mediated TER decrease, impairment of ZO-1 barrier protein, and induction of TNF-α and IL-8 (Fig. 6A–C). Removal of CS resulted in partial blocking of gp120-mediated TNF-α induction, which resulted in partial ZO-1 delocalization, but no significant decrease on TER. Treatment with phospholipase C in the presence or absence of gp120 decreased TER and affected the viability of the epithelial monolayers (data not shown).

Further studies showed that addition of exogenous HS in the presence or absence of cellular HS enhanced the ability of gp120 to induce cytokine production in primary GECs (Supplemental Fig. 2). These results indicate that HS is an essential cofactor in gp120-mediated signaling through TLR2 and TLR4.

**FIGURE 2.** Specificity of HIV-1 gp120 for induction of proinflammatory cytokines and chemokines from primary GECs. (A) Primary endometrial GECs were exposed to HIV-1 or gp120, and the time kinetics of apical and basolateral TNF-α and IL-8 production were measured from 1–16 h postexposure. Cytokines were analyzed by multianalyte bead assay. (B) Confluent primary GECs were exposed to mock infection (media), HIV-1, gp120, env-deleted HIV-1 (env-), or UV-inactivated HIV-1 for 24 h, and supernatants were analyzed for TNF-α or IL-8. (C) To determine whether these were gp120-specific responses, confluent primary GECs were exposed for 24 h to mock infection, HIV-1, gp120, env-deleted HIV-1 (env-), or UV-inactivated HIV-1 with or without anti-gp120 neutralizing Ab. TNF-α and IL-8 production in apical supernatants is shown. Significance shown in each graph is relative to the respective mock-infected group for each experiment. Data shown are representative of three to five separate experiments from individual tissues with similar results and represents mean ± SEM of triplicate cultures of the representative tissue. *p < 0.05, **p < 0.01, ***p < 0.001.
Soluble gp120 and HS can bind TLR2 or TLR4

Although gp120 is known to have four HS binding sites (37, 38), it is not known whether gp120 or HS can bind to TLR2 or TLR4, as suggested by our results. To further assess this, we developed bead-based binding assays. To standardize the assay, polystyrene beads were coated with soluble TLR4 or gp120, and efficient protein coating was verified by flow cytometry (Fig. 7A). Next, binding assays were conducted by coating beads with soluble TLR2, TLR4, or HS and subsequently incubating them with soluble gp120. Using a gp120 detection Ab, we observed that gp120 was specifically bound to these receptors (Fig. 7B). The binding specificity of gp120 to TLR4 was also supported by a direct enhancement in mean fluorescence intensity, as increasing gp120 concentrations were used (Fig. 7C). Furthermore, we also saw that TLR4 was specifically bound to HS (Fig. 7D). BSA-coated beads were used as controls to demonstrate the specificity of gp120 and TLR4 binding. Overall, these experiments indicate that HIV-1 gp120 can bind to TLR2, TLR4, or HS on host epithelial cells. gp120 and HS are required and sufficient for TLR-mediated signaling

To confirm that HS and gp120 were necessary to induce cytokine signaling in epithelial cells via TLR2 and TLR4 pathways, we used an artificial signaling expression system in the kidney embryonic cell line HEK293, which has been extensively used for testing TLR function (38, 39). HEK 293 cells were transfected with an NF-κB–luciferase reporter plasmid and stimulated with increasing doses of gp120 or known TLR ligands (positive control). gp120 induced significant NF-κB activation, in a dose-dependent manner, compared with mock treatment (Fig. 8A). In TLR2-transfected cells, NF-κB activation was comparable to that seen in response to Pam3CSK4 stimulation at the highest dose of gp120. HEK293 cells transfected with NF-κB–luciferase and stimulated with gp120 or Pam3CSK4 in the absence of TLR2 did not show any NF-κB activation (Supplemental Fig. 3). Transfection of HEK293 with CD14 in addition to TLR2 further enhanced NF-κB activation. A similar dose-dependent enhancement of NF-κB activation was seen when HEK293 cells were transfected with TLR4 and NF-κB reporter plasmids (Fig. 8A, right panel). Similar to FimH (23), we found that gp120 could also induce NF-κB activation through TLR4, even in the absence of CD14 and MD2.

To determine if HS was playing an essential role in gp120-mediated NF-κB activation via TLRs, transfected HEK293 cells were pretreated with heparinase III prior to exposure with gp120, Pam3CSK4, or FimH (Fig. 8B). Removal of HS resulted in gp120 not being able to stimulate NF-κB activation, but did not affect NF-κB stimulation by either Pam3CSK4 or FimH, indicating HS is essential for gp120-mediated signaling. Addition of exogenous HS in the absence of gp120 did not result in NF-κB activation, indicating that induction required gp120 in the context of HS. These results confirm that HS and TLR2/TLR4 are essential for NF-κB transduction and proinflammatory cytokine induction in transfected HEK 293 cells produced in response to HIV-1 gp120.
GECs induced TNF-α in response to gp120 present in semen samples

To determine the relevance of TLR activation in primary GECs following gp120 exposure to heterosexual transmission, we examined the effect of gp120 added to either SP from HIV-1-uninfected or infected individuals on primary female GECs. Confluent monolayers of primary GECs were exposed to 1:6 diluted uninfected SP or uninfected SP containing known amounts of recombinant gp120 protein. The dilution of SP was based on our previous studies, so that SP did not compromise the viability of GECs (28). Recombinant gp120 protein was used for comparison. Results indicated that GECs responded to gp120 present in SP in a dose-dependent manner, inducing significant levels of TNF-α, similar or slightly higher than the levels seen with gp120 alone (Fig. 9A). Furthermore, when GECs were exposed to HIV-1–infected SP, TNF-α was upregulated by two-fold compared to uninfected SP exposure (Fig. 9A). Because infected SP contains other proinflammatory cytokines that in turn induce inflammatory responses from GECs (28), we next determined whether addition of gp120 to AI SP, which contains the maximum amount of inflammatory cytokines, would have an additive effect on the induction of TNF-α from primary GECs. Confluent primary GECs monolayers were exposed

FIGURE 4. Neutralization of TLR2 and TLR4 pathways prevents the induction of proinflammatory cytokines and epithelial barrier breakdown by gp120. GEC monolayers were mock treated (media) or treated with gp120, Pam3CSK4, or FimH in the presence or absence of neutralizing Abs against TLR2, TLR4, TLR5, or isotype control Abs. Supernatants were collected and analyzed for TNF-α (A) and IL-8 (B) at 24 h postexposure, or GEC TER (C) was measured at pretreatment (0 h) and 24 h postexposure and expressed as a percent of pretreatment TER. To further measure barrier function, blue dextran dye leakage was measured across epithelial monolayers (D). Blue dextran in tandem with gp120 was added to primary GECs in the presence or absence of TLR neutralizing or isotype control Abs. At 24 h postexposure, basolateral supernatants were collected and absorbance was measured and calculated as a percent of apical blue dextran absorbance at 0 h. Significance shown in each graph above is relative to the respective mock-infected group for each experiment. Data shown represent mean ± SEM of the triplicate cultures from the representative tissue from three separate experiments. (E) Monolayers were fixed at 24 h post–gp120 treatment in the presence or absence of neutralizing Abs or isotype controls, and immunofluorescent staining for ZO-1 was performed. The corresponding Z-stack series are featured below each panel and show ZO-1 (green) and nuclei staining (red). Images were captured by a laser-scanning confocal microscope. Images are representative of one of three separate experiments with similar results. Original magnification ×1260. *p < 0.05, **p < 0.01, ***p < 0.001.
on barrier disruption. matory factors present in infected SP can add to the effect of gp120 matory responses in the presence of SP. Further, other proinflam-
uninfected SP spiked with recombinant gp120 and infected SP (Fig. 9B). The direct effect on the epithelial barrier was seen by production to comparable levels observed with pooled SP alone
TNF-α recombinant gp120 produced an additive effect on induction of from GECs, exposure to a combination of pooled SP with
a addition to infected SP and recombinant gp120 reduced TNF-
mechanism by which HIV-1 surface gp120 can be recognized by

Discussion
In the current study, we provide evidence for a novel intracellular mechanism by which HIV-1 surface gp120 can be recognized by innate pattern-recognition receptors, specifically TLR2 and TLR4, present on the female upper genital tract (endometrial and endocervical) epithelium. HS, a noncanonical attachment receptor for HIV-1, was found to be indispensable for gp120-mediated induction of TLR signaling in GECs. This interaction among HIV gp120, TLR2/4, and HS resulted in the activation of intracellular NF-κB pathway, leading to downstream upregulation of proinflammatory cytokines and chemokines, including TNF-α. Upregulation of these inflammatory factors was associated with tight junction disruption and loss of barrier function. Although binding interaction between gp120 and HS has been well described in previous studies, this is the first report, to our knowledge, that shows that gp120 can also bind directly, in a dose-dependent manner, to TLR2 and -4. Based on this, we propose that a trimolecular complex composed of gp120, HS, and TLR2 or TLR4 is required to activate the innate proinflammatory cytokine cascade in GECs in the context of an HIV infection.

Although this study is the first one, to our knowledge, to describe this mechanism in detail, induction of cytokines in epithelial cells by HIV-1 has been reported by other studies. Li and colleagues (41) showed that in response to R5 tropic HIV-1, human vaginal epithelial cell cultures produced significant amounts of chemokines MIP-3α and IL-8, whereas Fanibunda et al. (42) showed that genital epithelial cell gene signatures were changed in response to gp120, resulting in upregulated gene expression of TNF-α and the chemokines CXCL1 and CXCL8. Neither of these studies examined the mechanism by which induction could take place or whether the inflammatory factors had any effect on epithelial barrier functions.

The interaction between HIV-1 and the innate immune system is not well understood and has only recently started to garner attention (43). gp120 itself has been linked to immune activation of brain endothelial cells leading to the disruption of the blood–brain barrier and activation of LFA-1 on CD4+ T cells, enhancing their susceptibility to HIV-1 (44, 45). It is interesting to speculate whether innate recognition and activation of TLR pathways in response to gp120 could occur in DCs, which are also equipped for innate sensing, like epithelial cells. So far, to the best of our knowledge, HIV-1 has not been shown to activate DCs, although a previous study suggested that recognition of HIV-1 viral ssRNA by TLR7/8 on plasmacytoid DCs and monocytes may partially contribute to immune activation seen in HIV-infected individuals (43). Other studies have shown that binding of gp120 by DC-specific ICAM-3–grabbing nonintegrin can lead to suppression of IFN-α and apoptosis signal–regulating kinase 1–dependent cell death (46, 47). A recent report by Manel and colleagues (48) found that monocyte-derived DCs were unable to respond to HIV-1 in the absence of a productive infection, but when replication could be artificially induced, type I IFN responses were produced. This response was dependent on the interaction between newly synthesized HIV-1 capsid proteins with cellular cyclophilin A and the subsequent activation of the transcription factor IRF3. Although we did not investigate type I IFN production in this study, the proinflammatory responses seen in this study were not dependent on viral replication, because UV-inactivated virus and recombinant gp120 alone could induce these responses. One reason for such distinct responses among DCs and GECs could be differences in glycosaminoglycan chain expression on the cell surface of these cells, which could account for differences in recognition of HIV-1 and subsequent induction of innate responses. For example, CS is the major glycosaminoglycan chain at the surface of mononuclear lineages (49), including human monocyte-derived DCs, whereas HS moieties outnumber CS moieties (50) in the human female genital tract. Based on our results, HS is critical for the recognition of HIV-1 gp120 by GEC and activation of TLR pathway, whereas CS does not appear to act in a similar manner. Altogether, these results

![FIGURE 5. Neutralization of TLR2 and TLR4 on cell-surface blocks gp120-mediated NF-κB activation and nuclear translocation. Primary GECs were exposed to gp120 or mock treatment (media) for 1 h in the presence or absence of neutralizing Abs against TLR2, TLR4, TLR5, or isotype controls. Cells were fixed, and immunofluorescent staining for the NF-κB p65 subunit was performed. Nuclear counterstaining, as seen in red, was achieved using propidium iodide. Images were captures by a laser-scanning confocal microscope. Images are representative of one of three separate experiments with similar results. Original magnification ×1260.](http://www.jimmunol.org/)

to pooled SP from AI men in the presence or absence of exogenous recombinant gp120 with or without a gp120-neutralizing Ab. Mock infection, recombinant gp120 alone, or pooled SP from AI men containing exogenous recombinant gp120 and SP containing isotype control Ab were used for comparison. Although both pooled SP from AI men and gp120 alone increased TNF-α production from GECs, exposure to a combination of pooled SP with recombinant gp120 produced an additive effect on induction of TNF-α from GECs (Fig. 9B). Incubation with anti-gp120 Ab in addition to infected SP and recombinant gp120 reduced TNF-α production to comparable levels observed with pooled SP alone (Fig. 9B). The direct effect on the epithelial barrier was seen by a significant disruption of tight junctions by exposure to both uninfected SP spiked with recombinant gp120 and infected SP (Fig. 9C). These results indicate that gp120 can induce inflammatory responses in the presence of SP. Further, other proinflammatory factors present in infected SP can add to the effect of gp120 on barrier disruption.
suggest that each cell type expresses a distinct repertoire of cell-surface molecules and therefore likely engages in unique interactions with HIV-1, resulting in activation of differential signaling pathways and inflammatory responses.

The relevance of gp120-mediated TLR activation in heterosexual transmission was examined in experiments in which gp120 was found to exert the same effect in the context of semen. Despite the fact that the majority of HIV transmission takes place in the context of semen, the role of semen in HIV infection is not clearly understood. There is conflicting information whether semen inhibits or facilitates HIV infection (51–53). Our recent work showed that SP from both HIV-infected and uninfected men induced inflammatory responses in the epithelial cells of the FRT. However, higher levels of TGF-β1 in SP correlated with decreased proinflammatory cytokine production by GECs (30). Because semen can induce both inflammatory and immunoregulatory responses, we tested the effect of gp120 added to uninfected SP as well as SP from infected individuals. The results show that both SP spiked with gp120 and SP from HIV-infected individuals induced significant TNF-α induction from GECs and led to disruption of tight junctions (Fig. 9). Because SP from HIV-1-infected men contains proinflammatory cytokines that could in turn induce TNF-α from GECs, we examined the effect of combination of gp120 and infected SP and found an additive effect on the induction of inflammatory cytokines from GECs when the two are combined. This implies that physiologically, a combination of gp120 along with inflammatory cytokines present in infected semen could work together to enhance barrier disruption in FRT. In light of this, it would be interesting to determine if other sources of inflammation such as bacterial and viral coinfections and/or infected SP could exacerbate HIV-1 gp120 effects or, alternatively, effectively lower the amount of virus needed for barrier disruption.

Although the current study demonstrates that HIV-1 gp120 present in semen could directly activate inflammatory innate responses in GECs, the clinical significance of this pathway needs...
Whether gp120 concentrations comparable to those used in this study are present in SP is a contentious issue (54). The source of gp120 in semen could be from infectious, intact virions or as soluble gp120 shed from viral particles or infected cells. Soluble gp120 has been associated with a variety of biological activities and has been detected (~10 ng/ml) in the circulation of acutely or chronically infected individuals (14). Santosuosso et al. (55) showed that concentrations of gp120 >300 ng/ml could be detected in secondary lymphoid tissues obtained from HIV-infected subjects, even when gp120 was undetectable in

**FIGURE 7.** HIV-1 gp120 binds to TLR2, TLR4, and HS-coated microbeads. (A) The specificity of gp120, TLR4, and TLR2 coating on polystyrene microbeads was determined by flow cytometry using biotinylated anti-gp120, anti-TLR4, and anti-TLR2 Abs. Soluble TLR2, TLR4, and HS-coated beads were incubated for 1 h with gp120 (B), and binding was determined by biotinylated anti-gp120 Ab (C). Binding specificity of gp120 was confirmed by adding different doses of gp120 to TLR4 microbeads. (D) Soluble TLR4 was added to HS-coated beads, and TLR4 binding was determined by biotinylated anti-TLR4 Ab. In all experiments, BSA-coated beads were used as controls to establish positive staining of gp120 and TLR4 bead binding. Nonshaded peak represents unstained beads, gray-shaded peak represents background staining by streptavidin PE-Cy7 alone without primary Ab, and the black peak represents staining in the presence of primary and secondary Abs. Data shown are representative of three separate experiments with similar results.

**FIGURE 8.** gp120 activates NF-κB through TLR2 and TLR4, facilitated by HS. (A) HEK 293 cells were transiently transfected with an NF-κB-luciferase reporter plasmid and a TLR2 (left panel) or TLR4 (right panel) expression plasmid, with or without a CD14 expression plasmid, and 24 h posttransfection were exposed to treatment (media), Pam3CSK4 (Pam3), FimH, LPS, or gp120. At 48 h posttreatment, cells were disrupted, and fold increase in luciferase activity was measured in cells as a readout for NF-κB activation using commercial luciferase kits. (B) Alternatively, transiently transfected HEK 293 cells were treated with, or without heparinase III (HPIII) and/or exogenous HS for 1 h prior to gp120 stimulation. At 48 h posttreatment, cells were disrupted, and fold increase in luciferase activity was measured in cells as readout for NF-κB activation. Bars represent mean ± SEM of triplicates. Significance shown in each graph above is relative to the respective mock treated group for each experiment. Data shown are representative of three (A) or two (B) separate experiments with similar results. **p < 0.01, ***p < 0.001.
their blood plasma. Such high local concentrations of gp120 could be relevant in light of studies showing compartmentalized viral shedding between blood and semen seen in HIV-infected men, which could indicate a local reservoir of HIV-1 in the male genital tract (56–58). Our attempts to directly measure gp120 in SP of HIV-1–infected men have given varied results and proven to be technically challenging. Nevertheless, the mechanism shown in the current study provides a possible physiological scenario in which exposure to HIV-1 or soluble gp120 from semen from infected men could directly initiate immune activation.

The recognition of HIV-1 gp120 by GECs has important implications for HIV-1 susceptibility and pathogenesis. The innate recognition and inflammatory response of GECs could inadvertently provide an advantage to HIV-1. The direct disruption of tight junctions could allow paracellular leaking of HIV due to loss of mucosal barrier and be the source of HIV infection in the female upper genital tract. Although the exact mechanism is unclear, experimental studies in nonhuman primate models provide strong evidence that the female upper genital tract, particularly the endocervix, is a susceptible site for HIV-1 infection (3). Furthermore, our studies provide an explanation for the etiology of mucosal barrier disruption during the acute phase of HIV-1 infection, which has been associated with chronic immune activation, a hallmark of HIV disease progression (10, 11). Microbial translocation across the intestinal and possibly genital mucosa is considered to be the most likely reason for increased immune activation and inflammation during HIV-1 infection (9, 59). Activated T cells in the intestinal mucosa have been cited as the source of proinflammatory cytokines that breach the mucosal barrier in the gut (9, 60, 61). Our results show that HIV-1 gp120 directly induces an inflammatory response in GECs and provides an alternative explanation of a direct mechanism of barrier disruption and microbial translocation early after exposure to HIV (7).

In conclusion, this study provides evidence for the first time, to our knowledge, that gp120 can act as a TLR ligand on GECs in the presence of HS. This signaling complex leads to activation of TLR2 and TLR4 pathways, NF-κB activation, and subsequent induction of proinflammatory cytokines. Proinflammatory cytokines, specifically TNF-α, induce destabilization and disruption of tight junction proteins, loss in barrier function, and increased barrier permeability. Subsequently, both viral and bacterial translocation can take place across the epithelial monolayers. Altogether, these studies suggest that the initial innate recognition of HIV-1 could result in barrier loss and initiation of immune activation that could be the first step in chronic immune activation, a hallmark of HIV-1 pathogenesis. Strategies to prevent barrier loss following exposure to HIV-1/gp120 could provide the basis for prophylactic treatments for prevention of immune activation during HIV-1 infection.
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Disclosures

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