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Herpes Simplex Virus Type 2–Infected Dendritic Cells Produce TNF-α, Which Enhances CCR5 Expression and Stimulates HIV Production from Adjacent Infected Cells

Valerie Marsden,*† Heather Donaghy,*, Kirstie M. Bertram,*† Andrew N. Harman,* Najla Nasr,*, Elizabeth Keoshkerian,‡ Steven Merten,§ Andrew R. Lloyd,‡ and Anthony L. Cunningham*†

Prior HSV-2 infection enhances the acquisition of HIV-1 >3-fold. In genital herpes lesions, the superficial layers of stratified squamous epithelium are disrupted, allowing easier access of HIV-1 to Langerhans cells (LC) in the epidermis and perhaps even dendritic cells (DCs) in the outer dermis, as well as to lesion infiltrating activated T lymphocytes and macrophages. Therefore, we examined the effects of coinfection with HIV-1 and HSV-2 on monocyte-derived DCs (MDDC). With simultaneous coinfection, HSV-2 significantly stimulated HIV-1 DNA production 5-fold compared with HIV-1 infection alone. Because <1% of cells were dually infected, this was a field effect. Virus-stripped supernatants from HSV-2–infected MDDCs were shown to enhance HIV-1 infection, as measured by HIV-1–DNA and p24 Ag in MDDCs. Furthermore these supernatants markedly stimulated CCR5 expression on both MDDCs and LCs. TNF-α was by far the most prominent cytokine in the supernatant and also within HSV-2–infected MDDCs. HSV-2 infection of isolated immature epidermal LCs, but not keratinocytes, also produced TNF-α (and low levels of IFN-β). Neutralizing Ab to TNF-α and its receptor, TNF-R1, on MDDCs markedly inhibited the CCR5-stimulating effect of the supernatant. Therefore, these results suggest that HSV-2 infection of DCs in the skin during primary or recurrent genital herpes may enhance HIV-1 infection of adjacent DCs, thus contributing to acquisition of HIV-1 through herpetic lesions. The Journal of Immunology, 2015, 194: 4438–4445.

Herpes simplex virus type 2 (HSV-2) is the most common cause of genital herpes worldwide. Genital ulcer disease is increasingly associated with HSV-2 in developing countries, although in western countries this is mostly associated with HSV-1 in adolescents and young women (1, 2). A large number of epidemiological studies have demonstrated that prior or recent infection with HSV-2 markedly increases the sexual acquisition of HIV (HIV-1) >3-fold, both in the general population and those at high risk (3, 4). Recent initial acquisition of HSV-2, when viral shedding is greatest in the genital tract, shows an enhanced risk for HIV-1 acquisition compared with more remote initial HSV-2 infection (5). In African studies, acquisition of HIV-1 often occurs in a setting of an inflamed genital tract accompanying ulceration caused by sexually transmitted infections (2, 6). Doubts about this HSV-2–HIV-1 relationship, engendered by failure of antiviral suppression of recurrent genital herpes to affect HIV-1 acquisition, are now being understood in terms of the failure of antivirals to completely suppress HSV-2 shedding and epithelial microulcers (7–9). Conversely HIV-1–, HSV-2–infected individuals also shed HIV-1 more frequently than those without HSV-2, because of increased viral shedding through genital ulcers (10).

The mechanism of such HSV-2–enhanced HIV-1 transmission appears to be multifactorial. Genital ulcers associated with HSV-2 infection disrupt the protective upper layers of the epidermis (especially the stratum corneum) and produce a direct portal of HIV-1 entry into the epidermis, although not usually deeper into the dermis. Also, activated CD4 T lymphocytes and macrophages, which act as target cells for HIV-1 attachment and entry, infiltrate genital herpes lesions in epidermis and/or dermis (11–13). Increases in these HIV-1 target cell populations are evident in the genital mucosa even in the absence of clinical HSV-2 disease, during asymptomatic shedding, and also persist between lesions (14), suggesting that a persistent state of increased mucosal susceptibility is induced by HSV-2 infection. Indeed, HIV-1 has been shown to replicate to much higher levels in cells extracted from mucosa between lesions than from control mucosa.

The target cells for HIV-1 and HSV-2 in these lesions and how infection by one virus influences the other remains to be fully defined. Previous in vitro studies in HIV-1–transfected cell lines demonstrated that HSV-2 superinfection transactivates the promoter of HIV-1, enhancing HIV-1 production (15, 16). However, given the small number of HIV-1 virions transmitted through an HSV-2–infected or other sexually transmitted infection–infected and inflamed mucosa, such coinfection of individual cells within
lesions would be rare. HSV-2–HIV-1 interactions are more likely to occur as a field effect, with infection of adjacent cells individually by HSV-2 or HIV-1 influencing the other.

Dendritic cells (DCs) are present at the site of entry of both viruses, in the skin and mucosal surfaces, where they scout for foreign Ag, mature, and then migrate to stimulate T lymphocyte responses. Langerhans cells (LCs) in the epidermis of the strati

fied squamous anogenital mucosa are a target cell for productive infection by both HSV-2 and HIV-1 (17–20). They are major cell targets for HIV-1 in the uninfamed epimembrain, despite constituting only 1–2% of epidermal cells, and they extend their processes superficially into its upper layers to form a cellular network in contact with each other. They have been shown to take up HIV-1 in vaginal and foreskin explants ex vivo (18, 19) and also appear to be a target for SIV in macaque models (21). HSV-1/2 also infect the keratinocytes. Different subsets of DCs are found in the lamina propria underlying columnar epithelium of the endocervical and rectal/colonic mucosa and can extend processes into the lumen to take up HIV-1 (22, 23).

Separately, HIV-1 and HSV-1/2 have been shown to have profound and differing effects on the function of DCs, highlighting the importance of these cells in both infections. HIV-1 partially matures DCs (23), has no effect on their survival, and uses them for transfer to T cells. However, HSV-1/2 replicate in immature monocyte-derived DCs (MDDCs) and inhibit DC maturation before causing cellular apoptosis. HSV-2 also inhibits IL-12 production (24, 25). These findings have recently been confirmed (26). Apoptotic DCs are then taken up by bystanders and HSV-2 Ag presented to T cells (27). HSV-2 has also been shown to inhibit murine and human LC maturation, and induce apoptosis (17, 20).

In this study, we show that HSV-2 and HIV-1 coinfection of MDDCs in culture results in significantly enhanced HIV-1 infection and that this is a bystander effect mediated by cytokines, especially TNF-α stimulated by HSV-2 infection. The effect on enhanced HIV-1 infection of bystander MDDCs appears to be mediated by TNF-α–induced upregulation of CCR5 resulting in upregulated HIV-1 DNA levels. Such results suggest a mechanism for HSV-2 enhancement of HIV-1 acquisition immediately after the latter attains a foothold in the ulcerated epidermis of genital herpes lesions.

Defining the role of HSV-2 in fueling the HIV-1 epidemic at the level of the genital tract mucosa could lead to new and effective suppressive therapies that could help curb the number of new HIV-1 infections and/or reduce viral load.

Materials and Methods
Written and informed consent approved by the Western Sydney Local Health District human research ethics committee (HREC/09/WMED/307; Westmead Hospital, Sydney, NSW, Australia) was obtained from all patients undergoing breast reduction or abdominoplasty.

Generation of MDDCs and isolation of human LCs and other skin cells
MDDCs were generated from CD14 monocytes derived from whole blood as described previously (28–30). CD4 lymphocyte contamination was <0.1%, and monocytes were almost completely converted to CD14⁺, DC-SIGN⁺ DCs. Human LCs were extracted from human skin via collagenase digestion as described previously (23, 28) with 97% purity (31) (Supplemental Fig. 2). Human keratinocytes and fibroblasts were also isolated from the epidermis and dermis of these specimens, respectively, as previously described (32).

Virus preparations
Purified high-titer HIV-1_ada stocks in the order of 5 × 10¹⁰ 50% tissue culture-infective dose/ml were produced with the use of tangential filter concentration to eliminate extraneous cellular HIV DNA, proteins, and cytokines as described previously (29). HIV concentrations were determined as 50% tissue culture-infective dose value generated in TZM-b1 cells and by p24 gag ELISA (Beckman Coulter) as described previously (23, 33). Stocks of HSV-2 strain 186 were generated as described previously (34). The endotoxin levels of these virus stocks were less than the detectable limit of 0.005 U/ml or 0.0005 ng/ml (Limalus amebocyte lysate assay; Sigma-Aldrich).

MDDC infection
Immature day 5 MDDCs were infected with HSV-2 at a multiplicity of infection (MOI) of 0.1 to 3. Virus was left to adsorb for 2 h at 37°C; then the cells were washed and seeded at 1 × 10⁶/ml in RPMI 1640 supplemented with 10% human AB serum (RH-10) supplemented with IL-4 and GM-CSF.

For simultaneous coinfection studies, cells were infected with HSV-2 for 2 h as described above, then they were infected with HIV-1 (MOI 1 equal to 0.33 μg p24 Ag (Ag)/ 10⁶ cells) for 2 h followed by two washes with PBS.

For HSV-2 superinfection studies, cells were infected with HIV-1 stock (MOI 1) for 2 h and incubated for 4 d before infecting with HSV-2 as described earlier. The level of HIV infection of MDDCs was determined by flow cytometry for intracellular p24Ag at 96 h postinfection (hpi) and by quantitative PCR (QPCR) for HIV DNA.

Flow cytometry
Surface and intracellular flow-cytometry staining was carried out as described previously (29, 30, 33). For intracellular cytokine staining (ICS), cells were HSV-2 or mock infected for 2 h, washed, and cultured for 16 h with 5 μg/ml brefeldin A (Sigma-Aldrich). They were then stained with Live/Dead Near-IR (Life Technologies), surface labeled with FITC-conjugated anti-HSV2-gD, and then prepared for intracellular staining. Cells were permeabilized with cytofix/cytoperm (BD) for 15 min, washed, and stained with 1.25 μl brilliant violet 421–conjugated anti–TNF-α (BioLegend). Cells were acquired using a FACSCanto flow cytometer (BD) and analyzed using FlowJo (Tree Star, Ashland, OR) software. Results were analyzed after gating on MDDCs and live cells.

QPCR
QPCR was carried out as described previously to measure MDDC gene expression (28–30), HIV-1 DNA as late reverse transcription products (35), and HSV-2 ICP8 RNA (34) infection levels using a MX3005 QPCR machine (Stratagene, La Jolla, CA).

Serial filtration of supernatant
MDDCs were infected with HSV-2 (MOI 3) and cultured for 16 h. Cell-free supernatant was removed and spun in Amicon filter tubes with either a 100- or 30-kDa filter for 20 min at 5000 × g or a 3-kDa filter for 20 min at 7500 × g. Relevant filtrate fractions were incubated with MDDCs for 24 h to determine their effects on CCR5 expression.

TNF-α/TNFFR1 neutralization
MDDCs or filtered HSV2 supernatants were preincubated for 2 h at 37°C with neutralizing mouse mAbs to human TNFR1, TNFR2, or TNF-α (R&D), respectively. The MDDCs were washed, and cultured for 24 h with treated or untreated filtered HSV-2 supernatants. MDDCs were also cultured for 24 h with filtered mock supernatant as another control. Levels of CCR5 expression were determined by flow cytometry.

Luminex assay
Cytokine analysis of supernatants was conducted using a Human Cytokine 25-Plex Panel (Life Technologies) according to the manufacturer’s instructions and analyzed using a Bioplex-HTF with xMap Luminex detection technology.

Quantification of TNF-α and IFN-β by ELISA
ELISA analysis of supernatants was performed using Quantikine Human TNF-α Immunoassay kit (R&D) and VeriKine Human IFN-β ELISA kit according to the manufacturer’s instructions.

Results
HSV-2 infection in MDDCs increases the level of HIV-1 infection
To examine the effect of HSV-2 on HIV-1 infection in vitro, we infected MDDCs with both viruses, either simultaneously or as an HSV-2 superinfection.

For simultaneous infections, MDDCs were infected with HSV-2 at MOIs of 0.1 or 3 for 2 h. The cells were then washed before being...
infected with HIV-1 at an MOI of 1 and cultured at 37°C for 48 h. HIV-1 infection was measured as reverse-transcribed DNA and HSV-2 infection as early nonstructural ICP8 RNA, indicative of de novo replication, rather than uptake by MDDCs, which are highly endocytic. QPCR analysis for the infection level of each virus showed a significantly higher level of HIV-1 infection (as DNA) within the sample coinfected with HSV-2 at an MOI of 3 (but not 0.1) than for the HIV-1 infection alone (Fig. 1A). Conversely, the level of HSV-2 infection was significantly lower in samples coinfected with HIV-1 than in those with HSV-2 alone (Fig. 1B).

Flow-cytometry analysis for viral structural proteins was not possible for simultaneous infections because the kinetics and lytic effect of HSV-2 were too different from that of HIV-1 infection and the optimal time point for HSV-2 (24 hpi) too early for productive HIV-1 infection, as measured by p24 Ag. This was later done with superinfected experiments.

For HSV-2 superinfected experiments, MDDCs were infected with HIV-1 (MOI 1) and incubated for 4 d. These cells were then infected with HSV-2 (MOI 3) or mock treated and cultured for a further 24 h. There was no increase in the level of HIV-1 infection but a significant decrease in the level of HSV-2 (ICP8) RNA in the superinfected cultures (Fig. 1C and 1D). When expression of late-stage viral structural proteins by MDDCs was examined by flow cytometry, there was a decrease both in the proportion of HIV-1–infected cells, as determined by p24 Ag staining, and in the proportion of cells expressing HSV-2 gD (Fig. 1E).

This difference between the simultaneous and superinfected experiments strongly suggested that the effect of HSV-2 in increasing the level of HIV-1 infection was occurring early, probably before HIV-1 integration. In these superinfected experiments, where flow cytometry was possible, few (<1.5%) dually infected cells were observed within cultures infected with both viruses (Fig. 1E).

**HSV-2 infection results in upregulation of CCR5 on MDDCs**

In seeking an early effect of HSV-2 on HIV-1 coinfection, a comparison of surface HIV-1Rs on both mock and HSV-2–exposed MDDCs revealed that the level of surface CCR5 significantly increased after overnight HSV-2 exposure (Fig. 2A and 2B). The levels of cell-surface DC-SIGN, MR, and CD4 were not altered (data not shown). UV-inactivated HSV-2 had no effect on the surface expression of CCR5 (Fig. 2B).

The proportion of MDDCs expressing surface CCR5 (but not MFI per cell) was upregulated with increasing concentrations of HSV-2 and was maximal at 16 hpi at all MOIs (Fig. 2C and Supplemental Fig. 1). This increase in CCR5 post HSV-2 infection was also seen at the RNA level by QPCR, with a significantly higher level of CCR5 RNA after 24-h exposure, compared with mock-treated cells (Fig. 2D). HIV-1 infection had no significant effect on the level of CCR5 mRNA.

**Virus-free supernatant also leads to increased MDDC CCR5 and HIV-1 infection levels**

Because few dually infected cells were observed within coinfection experiments (Fig. 1E), we hypothesized that a field (bystander cell) effect was playing an important role in stimulating CCR5 and HIV-1 infection in MDDCs. To examine this, we centrifuged supernatant from HSV-2–infected MDDCs in an Amicon filter tube (100-kDa cutoff) to remove HSV-2 virions, but not cytokines, chemokines, and other soluble factors. This virus-free supernatant was then added to MDDCs for 24 h before infecting with HIV-1. Incubating MDDCs with this filtered supernatant achieved a very similar increase in the surface expression of CCR5 (Fig. 3A) and a subsequent increase in the HIV-1 infection level of these cells as measured by HIV-1 DNA and p24 Ag (Fig. 3B–D). Supernatant from HSV-2–infected MDDCs was passed through Amicon filter tubes with decreasing pore size (30 and 3kDa) to fully examine the supernatant for candidate cytokines. MDDCs were incubated with each of the supernatant fractions for 24 h and examined for CCR5 expression by flow cytometry. Once the supernatant had been passed through a 30-kDa filter, an increase in CCR5 expression (above mock-treated cells) was no longer seen. This re-
The molecular mass of the soluble factor increasing CCR5 expression is between 100 and 30 kDa (Fig. 3E).

Identification of CCR5-inducing cytokines in HSV-2–infected supernatants

These results focused our attention on cytokines or chemokines as soluble factors responsible for CCR5 upregulation. Therefore, a Luminex assay (25-plex Human proinflammatory cytokine panel) was performed on supernatants taken from HSV-2–infected MDDCs to identify these factors. Analysis revealed that TNF-α was the predominant cytokine within the supernatant (Fig. 4A). IL-4 and GM-CSF were also present, as expected, because these were added to differentiate monocytes into MDDCs. We also detected an increase in IP-10 and IL-8 within supernatants from HSV-2 infections, although to a far lower concentration than TNF-α. CCL3 and CCL4 showed a decrease in concentration within the HSV-2–infected supernatant compared with mock supernatant. In parallel, cultures of keratinocytes and fibroblasts were infected with HSV-2, and supernatants were collected at different time points.

FIGURE 2. HSV-2 infection results in upregulation of CCR5 expression by MDDCs. (A) Mock- and HSV-2–infected (MOI 3) MDDCs were assessed by flow cytometry for CCR5 expression after 24 h. A representative histogram is shown illustrating CCR5 surface expression on mock-infected cells and an increased expression on HSV-2–infected cells with an isotype control. (B) MDDC surface CCR5 expression measured by flow cytometry is increased by HSV-2 infection but not after inoculation with UV-HSV-2. Left panel shows percentage CCR5-expressing cells; right panel shows CCR5 MFI fold-change normalized to isotype (n = 4, mean ± SD; *p = 0.019, **p = 0.014). (C) Kinetics of CCR5 surface expression from 8 to 48 h post HSV-2 infection at different MOIs measured by flow cytometry (n = 3, mean ± SD). (D) HSV-2–infected MDDCs show significant increase of CCR5 RNA with an MOI of 3 at 24 hpi, quantified by QPCR (n = 3, mean ± SD; *p = 0.0002).

FIGURE 3. Supernatants from HSV-2–infected MDDCs stimulate CCR5 expression and HIV-1 infection in other MDDCs. (A) Virus-free supernatant stimulated MDDC CCR5 expression. Left panel as a percentage of CCR5 expression; right panel as CCR5 MFI fold-change (normalized to isotype). Incubation of MDDCs with filtered HSV-2 supernatant significantly increased CCR5 surface expression compared with mock-treated cells (n = 3, mean ± SD; *p = 0.033, **p = 0.03). (B) Virus-free supernatant increased intracellular HIV-1 DNA production by MDDCs. HIV-1 DNA levels measured by QPCR in HIV-1–infected and mock-infected MDDCs at 96 hpi. HIV-1 DNA levels were significantly higher in HIV-1–infected MDDCs when cells were preincubated with filtered supernatant than in mock-treated cells (n = 3, mean ± SD; *p = 0.027). (C and D) Virus-free supernatant also stimulated increased HIV-1 p24 Ag production in MDDCs. Representative FACS plots (C) and mean data (D) of p24 Ag in HIV-1–infected and mock-infected MDDCs at 96 hpi after pretreatment or not with filtered HSV-2 supernatants. There was a marked increase in the proportion of MDDCs expressing p24 Ag when cells were pretreated with the HSV-2 supernatants (n = 3, mean ± SD; *p = 0.03). (E) Serial filtration of supernatant from HSV-2–infected MDDCs indicates that the CCR5 stimulatory activity is lost once this supernatant is passed through a 30-kDa filter. Supernatant from HSV-2–infected MDDCs was passed through Amicon filter tubes with decreasing filter pore sizes (100, 30, and 3 kDa). MDDCs were incubated with each of these supernatant fractions for 24 h and examined for CCR5 expression by flow cytometry (n = 3, mean ± SD; *p = 0.014).
with HSV-2 at the same MOI and time point. No TNF-α or other relevant cytokines were detected in the supernatant (data not shown).

In MDDCs, the cellular RNA levels of key cytokines known to induce CCR5 (and control IL-1β) were quantified by QPCR (36). Our results indicated that TNFA was the only cytokine known to induce CCR5, which showed upregulation of RNA expression in early HSV-2 infection (Fig. 4B). Furthermore, we examined our previously published database of HSV-2–stimulated gene expression in MDDCs (29) and found no other likely cytokine or chemokine candidates (although HIV-1 replication modulating IFN-β was stimulated). Microarray data are available through the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32648) under accession number GSE32648. Then, using intracellular cytokine staining, we demonstrated that TNF-α is produced within HSV-2 Ag+ MDDCs 24 hpi (Fig. 4C and 4D).

**TNF-α is the major CCR5-inducing cytokine in HSV-2–DC supernatants**

To confirm that TNF-α increases CCR5 expression, we performed blocking experiments using neutralizing Abs against TNF-α itself and also against its cellular receptors TNFR1 and TNFR2. The surface expression of CCR5 was examined by flow cytometry on either mock treated MDDCs or MDDCs preincubated for 2 h at 37°C with neutralizing mouse mAb to human TNFR1 or TNFR2. Cells were washed and cultured for 24 h with filtered HSV-2 supernatant with or without anti–TNF-α. Highly significant inhibition was observed using anti–TNF-α and anti-TNFR1 Abs (Fig. 4E). No block was observed using anti-TNFR2.

**Live and UV-inactivated HSV-2 have discordant effects on MDDC and LC production of TNF-α and IFN-β**

Because HSV-2 can stimulate MDDCs and LCs to produce IFN-β, which could have opposite effects on HIV-1 infection, we examined the relative levels of TNF-α and IFN-β produced by MDDCs and also in LCs after exposure to live and UV-inactivated HSV-2 in parallel. Reciprocal effects on the two cytokines were observed for HSV-2 and UV-HSV-2, and were similar in both MDDCs and LCs. Live HSV-2 stimulated TNF-α to a significantly higher degree than UV-HSV-2, but stimulated IFN-β to significantly lower levels than UV-HSV-2 (Fig. 5A and 5B). The effects of HSV-2 infected DCs producing TNFα and the subsequent increase in HIV infection are summarized in Fig. 6.

**Discussion**

In this study, we showed that after coinfection of MDDCs with HIV-1 and HSV-2, each affected the replication of the other. The moderate inhibitory effect of HIV-1 on HSV-2 replication occurred early as HSV ICP8 RNA was inhibited. However, most notably, HSV-2 infection increased the level of HIV-1 replication 5-fold during the first cycle of replication at an early stage, as reverse-transcribed DNA. This effect increased with an increasing MOI of HSV-2. Nevertheless, HSV-2 superinfection of MDDCs that had already been infected for 4 d with HIV-1, when viral DNA is integrated, did not result in an increase in HIV-1 DNA or in the proportion of HIV-1 productively infected (p24 Ag+) cells in the culture. This strongly suggested that the HSV-2 stimulatory effect occurred early in the HIV-1 replication cycle, before integration. Very few dually infected MDDCs (<1.5%) were observed. This is not unexpected because only 5–10% of DCs were infected with HIV-1 or HSV-2 at 24 hpi and, as we have previously shown, at these MOIs, HSV-2 infection plateaus later at ~50–60% of the cell culture and for HIV-1 at 15–25% of MDDCs (25, 28). So this HIV-1 stimulatory effect had to be a field effect. This was confirmed by showing supernatants from HSV-2–infected DCs were stimulatory to HIV-1 production, as both HIV-1 DNA and p24 Ag were increased in freshly infected MDDCs. After filtering the supernatants, the HIV-1 stimulatory effect persisted in the absence of virus.
HSV-2 infection also increased CCR5 mRNA levels and the proportion of MDDCs expressing surface CCR5 protein in the culture as early as 8 hpi and peaking at 16–24 hpi, thus enhancing their susceptibility to HIV-1 infection. In myeloid cells, especially monocytes and macrophages, susceptibility and productive levels of infection are strongly determined by the levels of CD4 and CCR5 expression and by the binding affinity of HIV-1 envelope for CCR5 (37–39). A soluble factor within the supernatant of both MDDCs (*p = 0.008) and LCs (**p = 0.012) post HSV-2 infection (MOI = 3) for 24 h, and a smaller increase with UV-HSV-2 treatment (**p = 0.026 and ****p = 0.032, respectively) compared with mock treatment (n = 3, mean ± SD). (B) UV-inactivated, HSV-2–stimulated IFN-β secretion into supernatants of both MDDCs (*p = 0.021) and LCs (**p = 0.004) to a greater degree than live HSV-2 infection (MOI 3) (n = 3, mean ± SD; **p = 0.042 and ****p = 0.034, respectively).

FIGURE 5. Cytokine secretion by HSV-2–infected MDDCs and LCs. (A) Increased concentrations of TNF-α were observed in the supernatants of both MDDCs (*p = 0.008) and LCs (**p = 0.012) post HSV-2 infection (MOI = 3) for 24 h, and a smaller increase with UV-HSV-2 treatment (**p = 0.026 and ****p = 0.032, respectively) compared with mock treatment (n = 3, mean ± SD). (B) UV-inactivated, HSV-2–stimulated IFN-β secretion into supernatants of both MDDCs (*p = 0.021) and LCs (**p = 0.004) to a greater degree than live HSV-2 infection (MOI 3) (n = 3, mean ± SD; **p = 0.042 and ****p = 0.034, respectively).

To identify the soluble factor(s) capable of stimulating CCR5 expression, we lysed and analyzed HSV-2–infected MDDCs by QPCR for cytokines known to induce such an effect (42), and by DNA microarray for enhanced cytokine and chemokine gene expression using our previously published database (29). The supernatants from HSV-2–infected DCs were also analyzed by Luminex assay for such cytokines and chemokines and by ELISA for IFN-β. DNA microarray, QPCR analysis, Luminex, and ELISA assays showed enhanced expression of several cytokine and chemokine genes, including IFN-β, but by far the most up-regulated cytokine was TNF-α, expressed as RNA as early as 4 hpi. Luminescent analysis confirmed that TNF-α was the major cytokine produced, with minor enhancement of CXCL10 (IP-10) and IL-8. TNF-α production in this setting was consistent with a previous report on HSV-2–infected MDDCs, although we found only low levels of IL-6 (26). Because TNF-α is a soluble trimer, with a total size of 51 kDa, it fits within the identified m.w. band. Intracellular cytokine staining was also used to demonstrate that TNF-α was produced by the infected cells within a HSV-2–infected MDDC culture. The enhancement of CCR5 expression after exposure to HSV-2 was significantly inhibited (65%) with both neutralizing Abs against TNF-α and its receptor TNFR1. The application of TNF-α–containing supernatants would be expected to induce partial MDDC maturation and delay the degradation of HIV p24 Ag in virus-containing compartments, which could confound using this test as a measure of de novo HIV replication. However, this was anticipated by performing the intracellular p24 Ag assays at 96 hpi, long after such Ag has decayed to negligible levels in mature MDDCs, as previously shown with much more potent inducing stimuli (40).

Because keratinocytes within the epidermis are the major in vivo target after HSV-2 exposure, we also examined whether they might contribute to this effect. However, by Luminex analysis, neither human epidermal keratinocytes nor human dermal fibroblasts produced any TNF-α postinfection with HSV-2 (data not shown). This is consistent with our previously reported data showing that HSV-2–infected keratinocytes are stimulated to produce β chemokines and low levels of IL-6, IL-10, and IL-12 only at 4–5 d postinfection, but not TNF-α at any time point (32). Furthermore, the late production of these cytokines from keratinocytes suggests that they are unlikely to modulate the rapid production of TNF-α by HSV-2–infected LCs. Nevertheless, another molecule LL-37 released by HSV-2–infected keratinocytes has also been reported to enhance HIV-1 infection of model monocyte-derived LCs (MDLCs) (43). These studies need to be confirmed with authentic isolated immature LCs because there can be marked genotypic, phenotypic, and functional differences between model and authentic epidermal LCs (28); for example, MDLCs, but not epidermal LCs, express mannose receptor and DC-SIGN. If confirmed, this would indicate that there are two different and possibly complementary ways in which the two major HSV-2–infected cell types in epidermis stimulate HIV-1 production by adjacent infected LCs.

Thus, the effects of HSV-2 infection of cultured MDDCs on uninfected bystander cell types provide useful models to examine the interaction of HSV-2 and HIV-1 during coinfection that may occur when HIV-1 enters a recurrent anogenital herpetic lesion during the period of active HSV-2 replication and spread, as...
summarized in Fig. 6. Studies in mice and humans suggest that the interaction of HSV-2–infected LCs with dermal DCs may both play a role in Ag processing and presentation to T cells (17, 20, 44, 45). The sequence of these events is clear in mice (17) and recently was confirmed in humans (20). However, in humans, dermal DCs, together with adjacent CD4 and CD8 lymphocytes, are also part of the upper dermal infiltrate that persists between recurrences of genital herpetic lesions. Single-cell suspensions of these dermal infiltrates from skin biopsies supported HIV-1 superinfection in vitro (13). In mice, MDDCs generated in vivo also infiltrate reparative lesions (45).

One of the major differences among MDDCs, dermal DCs, and epicutaneous LCs is their expression of different types of C-type lectin receptors, langerin on LCs and DC-SIGN and mannose receptor on MDDCs and dermal DCs (and all on MLDCs) (28). Indeed, de Jong et al. (46) have reported data suggesting UV-HSV-2 interacts with and downregulates langerin, which is then postulated to reverse langerin-mediated inhibition infection of LCs. This is the apparent opposite of the enhancing effect of DC-SIGN on infection of MDDCs (47, 48). However, other laboratories have reported langerin either enhances or has no effect on HIV-1 infection of LCs (49, 50). In our studies, we found that supernatants from HSV-2–infected MDDCs had no effect on langerin expression by LCs and DC-SIGN expression by MDDCs, respectively (data not shown). Furthermore, the HIV-1 stimulatory effect we observed was dependent on HSV-2 infection, not just binding/entry, because UV inactivated HSV-2 had no such effect. So the stimulatory effects we observed were separate from those reported with UV-HSV-2 (46) and specific to live HSV-2.

Our results showing no effect of HSV-2 superinfection on MDDCs, previously infected with HIV-1 for 4 d to increase HIV-1 DNA, are not contradictory to a previous report (26) where supernatants from HSV-2–exposed MDDCs (containing TNF-α and IL-6) were able to induce HIV-1 replication in the transformed U1 monocytoid cell line where the virus is integrated in every cell. This stimulatory effect of TNF-α on monocytoid cell lines such as U937 and U1 is well-known and operates through the NF-κB sites in the LTR (47). However, TNF-α has different effects on DCs infected de novo, for example, it induces maturation in MDDCs, which eventually reduces their susceptibility to productive HIV-1 infection (26, 48). The balance between inhibition of HIV-1 production by IFN-β and stimulation by TNF-α after live and UV inactivated HSV-2 stimulation also explains apparent differences from two previous reports (26, 46). One of these reports (46) discounted any effect of TNF-α in enhancing HIV-1 production by LCs, but this is explained by the other, which showed that MDDCs exposed to UV HSV-2 produced greater levels of IFN-β but lower levels of TNF-α than those exposed to infectious HSV-2 (26). We found similar effects on TNF-α and IFN-β in LCs and, importantly, no effect of UV-HSV-2 on HIV-1 coinfection; infectious HSV-2 was required.

Another mechanism of enhanced susceptibility to HIV-1 post HSV-2 infection has been suggested by Martinelli et al. (51). This study found that HSV-2 infection of MDDCs enhanced their production of retinoic acid, which mediated αβ2 upregulation in DC-T lymphocyte cultures. Integrin αβ2 allows lymphocytes to access gut tissue, a major site of HIV-1 replication. Rectal HSV-2 infection of macaques increased the frequency of αβ2+CD4+CD8 T lymphocytes within the blood and rectal tissue.

Thus, our study demonstrates a mechanism by which HSV-2 infection of skin/mucosa during initial, recurrent, or asymptomatic infection can contribute to increased production of coinfecting HIV-1 within the anogenital stratified squamous epithelium after its earliest penetration. The most likely scenario is that of infection of neighboring LCs with HIV-1 and HSV-2 (or HSV-2/1) in the epidermis after ulceration. However, this interaction could also occur with other DCs in the dermis during active HSV-2 infection of and transport by LCs into the dermis and then lead to infection of surrounding infiltrating CD4 lymphocytes.

These results may have implications for HSV-2 therapy and preventative strategies. Access of HIV-1 to recurrent herpes lesions is clearly highly risky for transmission and provides further incentive for the development of microbicides to prevent both HIV-1 and HSV-2 infection of skin/mucosal DCs and T cells.

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Disclosures

The authors have no financial conflicts of interest.

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