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Chapter 4

Herpes simplex virus-2 enhances HIV-1 susceptibility by affecting Langerhans cell function

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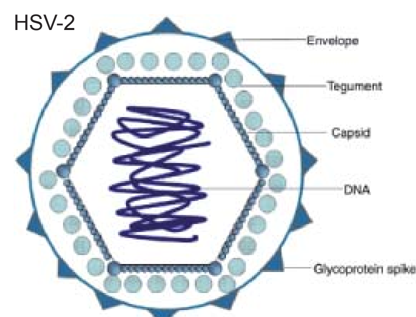

Revised manuscript submitted

Abstract

Genital herpes is the most prevalent viral sexually transmitted infection (STI) world-wide and is mainly caused by herpes simplex virus (HSV)-2. HSV-2 infection has been linked to enhanced HIV-1 susceptibility, even in the absence of clinical symptoms, but little is known about the molecular mechanisms involved. HIV-1 transmission mainly occurs via heterosexual transmission at genital mucosa and mucosal Langerhans cells (LCs) are involved in HIV-1 transmission. LCs form a tight network in mucosal tissues and efficiently capture and degrade HIV-1 through the C-type lectin Langerin, thereby preventing HIV-1 transmission.

We investigated the role of LCs in the enhanced HIV-1 transmission in the presence of viral STI such as HSV-2. Strikingly, low concentrations of HSV-2 strongly enhanced HIV-1 infection of LCs and subsequent HIV-1 transmission to T cells. Further analyses demonstrated that HSV-2 infection strongly decreased HIV-1 capture by Langerin, which increased HIV-1 infection of LCs. HSV-2 interfered with the anti-viral function of Langerin at two levels: HSV-2 infection decreased Langerin expression and HSV-2 competed with HIV-1 for Langerin binding. Furthermore, HSV-2 replication was not required, since both UV-inactivated HSV-2 and TLR-3 agonist Poly(I:C) similarly increased HIV-1 transmission by LCs.

We identified a mechanism by which HSV-2 enhances HIV-1 susceptibility even in the absence of clinical symptoms. Our data demonstrate that viral co-infections such as HSV-2 breach the protective function of LCs by activating LCs, which increases HIV-1 susceptibility. These data reinforce the importance of prevention of STIs such as HSV-2 to reduce the transmission of HIV-1.

Introduction

Over 30 million people world-wide are infected by HIV-1, the causative agent of AIDS and heterosexual transmission of HIV-1 is the primary route of infection (1). Numerous studies have demonstrated that sexually transmitted infections (STIs) enhance susceptibility to HIV-1 acquisition and transmission, even in absence of clinical symptoms (1-5). The most common viral STI acquired during sexual contact in the developing world is genital herpes (6), which is primarily caused by herpes simplex virus type 2 (HSV-2), although HSV-1 infection can also lead to genital herpes (7). Pre-existing HSV-2 infection is a risk factor for acquiring HIV-1 infection through sexual contact (1-4). Although it remains unclear how HSV-2 enhances susceptibility to HIV-1, several mechanisms have been proposed. The formation of pustules and ulcers by genital herpes can facilitate HIV-1 entry into mucosal tissues (8). In addition, mucosal inflammation by HSV-2 can cause influx of activated $CD4^+$ T cells, which facilitates HIV-1 infection and subsequent dissemination (9). Notably, HSV-2 can enhance HIV-1 susceptibility in the absence of apparent lesions (5) and HSV-2 causes inflammatory processes, including T cell influx, even in the absence of clinically apparent lesions. Furthermore, HIV-1 susceptible inflammatory cells persist for a prolonged period of time after HSV-2 infection has been cleared and the lesions have healed (10).

HSV-2 infection occurs via direct contact with infected lesions or body fluids and HSV-2 enters the body at mucosal tissues or through small lesions. Epithelial cells and keratinocytes are the primary target cells for HSV-2 but HSV-2 also infect neuronal and immune cells (11). Once infected, the virus causes life-long infection of the host by establishing latency in the neurons of the sensitive ganglia. Reactivation occurs periodically at times when the immune system is suboptimal, resulting in the formation of vesicles that break into ulcerations at the genital mucosa (8;12). The genital mucosa is crucial in sexual transmission of HIV-1 and co-infections with HSV-2 might affect the genital mucosa and thereby increase HIV-1 susceptibility. Genital tissues contain Langerhans cells (LCs), the main dendritic cell (DC) subset present in stratified epithelia and mucosal tissues (13). LCs are involved in HIV-1 dissemination; HIV-1 can infect LCs and infected LCs can subsequently migrate to the lymphoid tissues where they transmit the virus efficiently to T cells and thereby mediate infection of the host (14). However, we have recently shown that LCs have an anti-HIV-1 function (15). Under non-inflammatory conditions, LCs prevent HIV-1 transmission by efficient capture of invading HIV-1 via the C-type lectin Langerin, which targets the virus for degradation (15). Langerin prevents HIV-1 infection of LCs and thereby transmission of the virus from mucosal tissues to T cells in lymphoid tissues. Thus, LCs form a first line of defence against HIV-1 infection. However, inhibition of the anti-viral function of Langerin or inflammatory conditions allows infection of LCs by HIV-1, and infected LCs efficiently transmit HIV-1 to T cells (15;16). Therefore, we hypothesized that viral STIs such as HSV-2 affect the protective function of LCs and thereby increase HIV-1 susceptibility.

Here we show that HSV-2 productively infects LCs and increases HIV-1 transmission by LCs to T cells. We have identified two distinct effects of HSV-2 on the anti-HIV-1 function of LCs; HSV-2 induces down-regulation of Langerin expression and in addition HSV-2 virions inhibit HIV-1 binding to Langerin by direct competition with HIV-1. The reduced Langerin expression and function, decreases HIV-1 capture by LCs and the reduced Langerin function allows infection of LCs by HIV-1 and strongly increases HIV-1 transmission to T cells. Notably, active HSV-2 replication was not required, since UV-inactivated HSV-2 similarly increased HIV-1 transmission by LCs. Furthermore, our data show that TLR-3 agonist Poly(I:C) also increased HIV-1 transmission. Thus, co-infections with HSV-2 as well as double-stranded RNA viruses are major risk factors that affect the anti-HIV-1 function of LCs and thereby increase HIV-1 susceptibility. These data further support an important role for LCs in HIV-1 transmission in the presence of sub-clinical genital herpes and other viral STIs.

Results

HSV-2 productively infects human LCs

HSV-2 is one of the most common viral STIs (7) and is a risk factor for enhanced HIV-1 susceptibility (1-4). Therefore, we investigated whether HSV-2 infects LCs and thereby affects HIV-1 transmission. Both infectious HSV-2 and UV-inactivated HSV-2 (UV-HSV-2) were used to distinguish between effects caused by viral replication and through direct activation by pattern recognition receptors. Epidermal LC fraction was infected with different concentrations of HSV-2 for 18 hrs and cell-surface expression of HSV-2 glycoprotein gB was analysed to determine HSV-2 infection. gB is an envelope glycoprotein that is expressed as a late viral gene product (17). Both LCs and CD1a-negative keratinocytes were productively infected with HSV-2 in a concentration dependent manner (Figure 1A,B). HSV-2 did not significantly affect the viability of the LCs within 18 hrs (data not shown). HSV-2 infection of LCs increased with the viral load used: at 1x 10^5 p.f.u. approximately 10% of LCs were infected while at the highest viral load (10x 10^5 p.f.u.) up to 45% of LCs were infected (Figure 1B). We never observed more than 3% of gB-positive cells when LCs were incubated with similar concentrations of UV-HSV-2 (data not shown), demonstrating that the gB staining is due to productive infection of LCs and not to uptake of HSV-2.

To confirm productive HSV-2 infection, LCs were purified from the epidermal LC fraction and subsequently infected with HSV-2 or UV-HSV-2 and expression of HSV-2 thymidine kinase (tk) mRNA was measured by quantitative real-time PCR. In contrast to UV-HSV-2, HSV-2 infection induced transcription of *tk*, which increased over time and was dose dependent (Figure 1C,D). In addition, intracellular expression of HSV-2 immediate early protein ICP27 and late viral protein ICP5 was analysed by confocal microscopy. Both ICP27 and ICP5 were expressed by LCs after 24 hrs of infection with HSV-2 but not with UV-HSV-2 (Figure 1E; data not shown), Together these data demonstrate that LCs are productively infected by HSV-2.

HSV-2 and viral TLR agonists induce LC maturation

Next we investigated whether HSV-2 infection or viral TLR agonists induce LC maturation. Viral recognition is mainly mediated by TLR-3 for double-stranded RNA, TLR-7 and -8 for single-stranded RNA, and TLR-9 for unmethylated DNA Of the viral TLRs immature LCs express TLR-3 and -7, but little TLR-8 and no TLR-9 (18;19) Epidermal LC fraction (Figure 2A) was infected with HSV-2 or treated with UV-HSV-2 and TLR-3, -7 and -9 agonists Poly(I:C), Imiquimod, and bacterial CpG-containing oligonucleotides (CpG-ODN), respectively. LC maturation was determined by measuring co-stimulatory molecules CD80 and CD86 after 18 hrs by flow cytometry. HSV-2 infection of LCs in contrast to a mock infection control induced up-regulation of the co-stimulatory molecules CD80 and CD86 (Figure 2B and data not shown). UV-HSV-2 also induced up-regulation of the co-stimulatory molecules to a similar extent as infectious HSV-2 (Figure 2B). Poly(I:C) and to a lesser extent Imiquimod induced expression of CD80 and CD86, whereas CpG-ODN did not affect LC maturation (Figure 2B). Thus, both HSV-2 infection and viral TLR-3 agonist induce LC maturation.

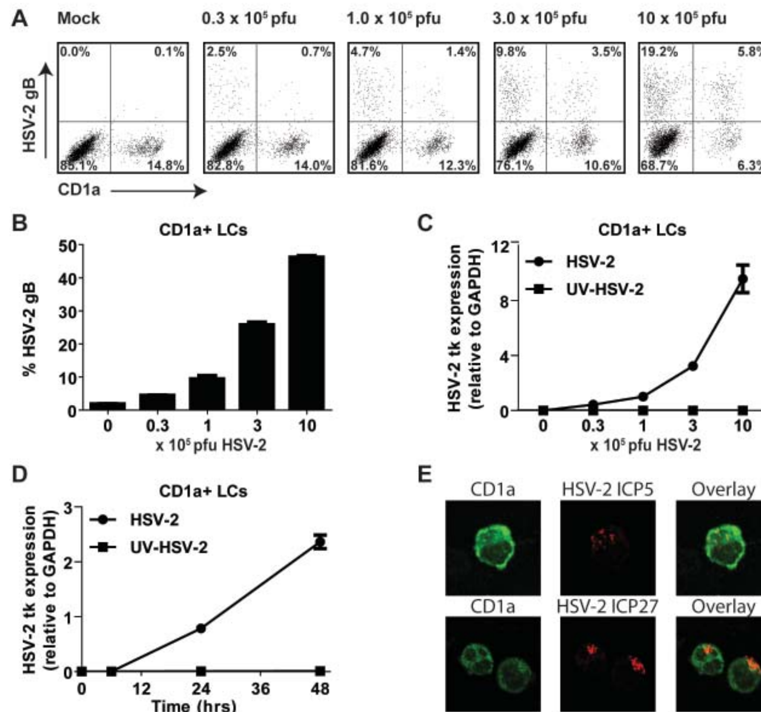

Figure 1. HSV-2 productively infects LCs. (A,B) Epidermal LC fraction was infected with different concentrations HSV-2 for 18 hrs. HSV-2 infection was detected by HSV-2 gB staining in flow cytometry. A double staining with CD1a was performed to select LCs. (A) Numbers represent the percentage of the total population. (B) Percentage of LCs that were infected with HSV-2. Error bars represent standard deviations of duplicates. These results were observed for three donors. (C,D) CD1a$^+$ LCs (1x 10^5) were isolated using MACS isolation and infected with HSV-2 or UV-HSV-2. mRNA expression of HSV-2 *thymidine kinase* gene was measured at different viral inputs (C) after 24 hrs, or at different time points (D) at 1x10^5 p.f.u.. Transcription was adjusted for *GAPDH* expression. (E) CD1a$^+$ LCs (1x 10^5) were incubated with HSV-2 (1x 10^5 p.f.u) for 24 hrs and stained for HSV-2 ICP5 and ICP27 (red) and CD1a (green). Images were taken using confocal microscopy at a magnification of 630x with a 4x zoom.

HSV-2 and viral TLR agonists decrease Langerin expresssion and increase HIV-1 infection

HIV-1 receptors CD4 and CCR5 are crucial for the infection of LCs with R5-tropic HIV-1, whereas in contrast, Langerin expression prevents infection of LCs (15). Therefore, we investigated the effect of HSV-2 and viral TLR agonists on the expression of CD4, CCR5 and Langerin. The expression of HIV-1 receptors CD4 and CCR5 was not affected by HSV-2, or any of the TLR ligands (Figure 2B). Notably, HSV-2 infection, UV-HSV-2 and Poly(I:C) strongly decreased Langerin expression, whereas Imiquimod only marginally decreased Langerin expression (Figure 2B).

The C-type lectin Langerin efficiently captures HIV-1 for degradation and thereby prevents HIV-1 infection of LCs (15). Therefore, we investigated whether the decreased expression of Langerin by HSV-2 and Poly(I:C) decreases HIV-1 capture. Primary LCs were treated with HSV-2, UV-HSV-2 and different TLR agonists

overnight and washed extensively before binding to HIV-1 gp120 was determined. As shown previously (15), HIV-1 gp120 interacted efficiently with immature LCs and the interaction was blocked by the competitive inhibitor of Langerin, mannan (Figure 3A), strongly suggesting that Langerin is the primary receptor for HIV-1. Notably, both HSV-2 infection and incubation with UV-HSV-2 significantly decreased HIV-1 binding to LCs in a concentration dependent manner (Figure 3A). Poly(I:C) also decreased HIV-1 binding to LCs, while Imiquimod or CpG-ODN did not alter HIV-1 binding (Figure 3A). Subsequent incubation with mannan reduced the binding to background level for all conditions (Figure 3A, data not shown). These data demonstrate that LC maturation by HSV-2 infection, UV-HSV-2 and TLR-3 agonists decrease HIV-1 capture by LCs, which might be due to down-regulation of Langerin expression.

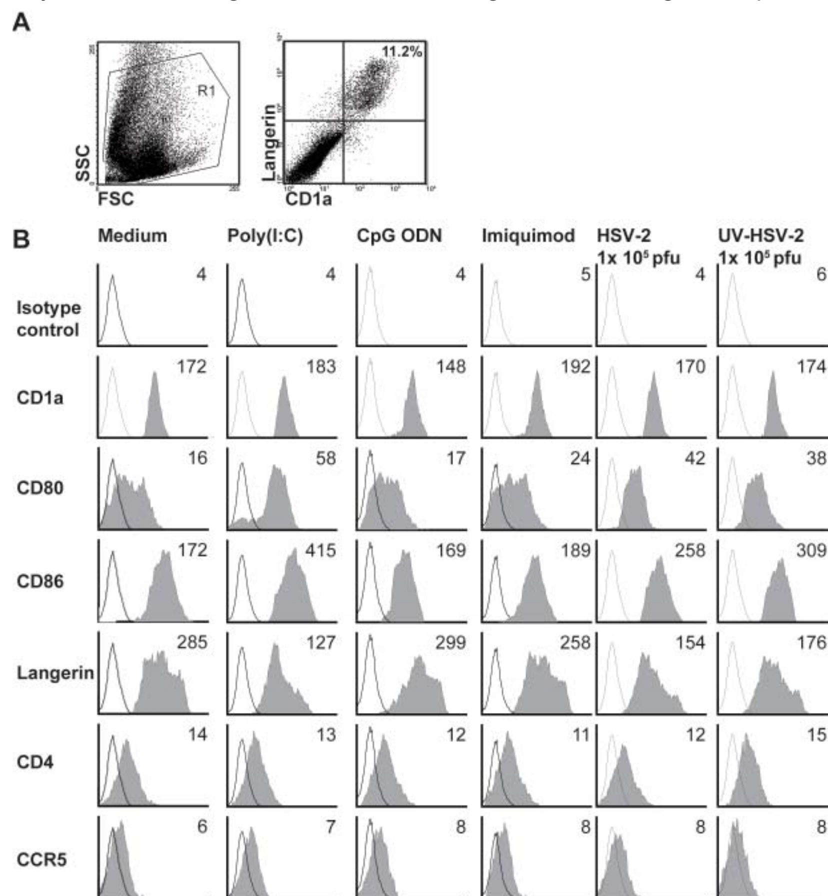

Figure 2. TLR agonists and HSV-2 induce maturation of LCs. (A) Epidermal LC fraction was isolated from human skin and LCs were analysed for CD1a and Langerin expression by flow cytometry. (B) Cells (1x10^5) were stimulated with Poly(I:C) (10 µg/ml), CpG ODN (10 µg/ml), Imiquimod (10 µg/ml), HSV-2 (1x10^5 p.f.u.) or UV- inactivated HSV-2 (UV-HSV-2) (1x10^5 p.f.u.). After 18 hrs, CD1a$^+$ LCs were analysed for the expression of co-stimulatory molecules CD80 and CD86, Langerin, and HIV-1 receptors CD4 and CCR5 by flow cytometry. Open histograms depict isotype control, filled histograms represent the depicted molecules. Mean fluorescent intensity is shown in the upper right corner. These results are representative of more than three donors.

Next, we investigated whether the decreased Langerin expression by HSV-2 and TLR-3 triggering increases HIV-1 infection. LCs were incubated overnight with HSV-2, UV-HSV-2 and viral TLR agonists, washed and infected with R5-tropic HIV-1. HIV-1 replication was determined after 6 and 24 hours by measuring Tat-Rev transcripts using quantitative real-time PCR (16). Notably, HSV-2, UV-HSV-2 and Poly(I:C) strongly enhanced HIV-1 transcription, in contrast to Imiquimod and CpG-ODN (Figure 3B,C). Thus, HSV-2 infection as well as stimulation with UV-HSV-2 and viral TLR-3 agonists enhances HIV-1 infection of LCs, which might be due to LC maturation and down-regulation of Langerin expression.

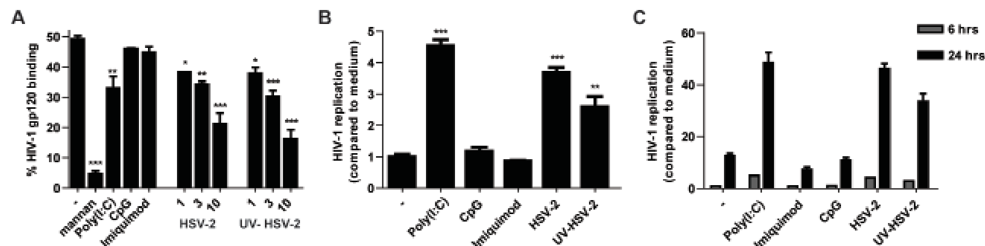

Figure 3. HSV-2 and TLR stimulation decreases HIV-1 capture and enhances HIV-1 infection of LCs. (A) Epidermal LC fraction was stimulated for 18 hrs with different stimuli and LCs were analysed for HIV-1 gp120 binding. Specificity was determined by inhibiting Langerin using mannan and EGTA. Graph depicts percentage of LCs that have captured HIV-1 gp120. Error bars represent standard deviations of triplicates. These results are representative for three donors. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (B,C) Epidermal LC fraction was stimulated for 20 hrs with different stimuli. Next, stimulated cells were incubated with R5-tropic HIV-1-NL4.3-BaL for 6 hrs (B,C) or 24 hrs (C). HIV-1 replication was determined by measuring tat/rev transcripts by quantitative real time PCR, and normalized for GAPDH expression. Medium condition was set at 1. Error bars represent standard deviations of duplicates. These results were observed for three donors. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

HSV-2 competes with HIV-1 for Langerin binding

Langerin has been shown to interact with different pathogens such as HIV-1, *Candida albicans*, and *Mycobacterium* (15;20-22). Therefore, we investigated whether HSV-2 interacts with Langerin and thereby competes with HIV-1 for the carbohydrate recognition domain of Langerin. Recombinant Langerin strongly interacted with HSV-2 in a concentration dependent manner (Figure 4A) as determined by the Langerin binding ELISA (23). The binding was specific for the carbohydrate recognition domain of Langerin, since it could be blocked by mannan and calcium-chelator EGTA. These data indicate that HSV-2 can compete with HIV-1 for the Langerin binding site. To investigate this in a cellular assay, LCs were pre-incubated with HSV-2 for 15 min and HIV-1 gp120 capture was measured by flow cytometry. The short incubation time excludes LC maturation and infection. Notably, pre-incubation of LCs with HSV-2 strongly reduced binding of HIV-1 gp120 to LCs (Figure 4B), demonstrating that HSV-2 blocks HIV-1 capture by directly competing with HIV-1 for Langerin. Thus, these data show that HSV-2 prevents HIV-1 capture by Langerin by down-regulation of Langerin expression as well as competition for the Langerin binding site.

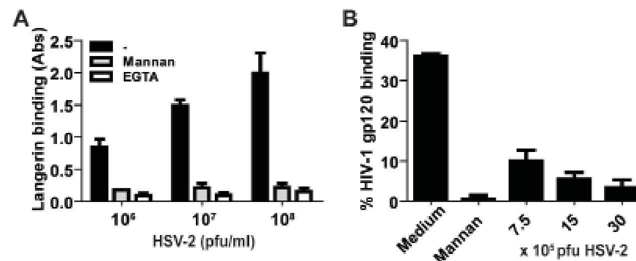

Figure 4. HSV-2 competes with HIV-1 for Langerin binding. (A) HSV-2 viral particles were coated onto an ELISA plate and binding was detected using recombinant Langerin. Mannan and EGTA pre-incubation indicate the specificity for Langerin. Error bars represent standard deviations of triplicates. These results were observed in three individual experiments. (B) LCs were pre-incubated with different concentrations of HSV-2 for 15 min before HIV-1 gp120 beads were added. The percentage of LCs that have bound HIV-1 gp120 is depicted. Error bars represent standard deviations of duplicates. These results are representative for three donors.

HSV-2 and Poly(I:C) enhance HIV-1 transmission by LCs to T cells

HIV-1 transmission by LCs to T cells is involved in dissemination of HIV-1 (15;16). Under steady-state conditions, LCs are refractory to HIV-1 infection through the protective function of Langerin (15). However, our data show that both HSV-2 and TLR-3 agonist Poly(I:C) increase HIV-1 infection of LCs by inducing LC maturation and affecting Langerin function. Therefore we investigated whether HSV-2 and TLR agonists increased HIV-1 transmission to T cells. Infectious HSV-2 could not be used in the transmission assays since HSV-2 also infects T cells and induces cell-death in the co-culture after several days (data not shown) even in the presence of HSV-2 inhibitor acyclovir. Epidermal LC fraction was treated with viral TLR agonists and UV-HSV-2 for 20 hours and washed extensively to remove the stimuli. Next, the stimulated LCs were incubated with R5-tropic HIV-1-eGFP for two hours, washed extensively and subsequently co-cultured with CCR5$^+$ Jurkat T cells (16). Viral transmission was followed over time by flow cytometry (16). LCs did not efficiently mediate HIV-1 transmission (Figure 5A,B) (15;16). Strikingly, both UV-HSV-2 and Poly(I:C) significantly enhanced HIV-1 transmission and this was consistent for different donors (Figure 5A,B). Imiquimod marginally enhanced HIV-1 transmission, however this was not statistically significant. CpG-ODN did not affect transmissioTo investigate whether the increased transmission by HSV-2 and Poly(I:C) was a direct effect on LC function or a bystander effect through activation of keratinocytes, LCs were purified from the epidermal single cell suspension by CD1a isolation. CD1a$^+$ LCs and CD1a$^-$ keratinocytes were stimulated with either UV-HSV-2 or Poly(I:C) overnight and subsequently infected with R5-tropic HIV-1-eGFP for 40 hours. Cells were washed extensively, CCR5$^+$ Jurkat T cells were added and HIV-1 transmission to T cells was measured over time. Strikingly, both Poly(I:C) and UV-HSV-2 strongly enhanced HIV-1 transmission by CD1a$^+$ LCs LCs, whereas no transmission was observed with keratinocytes (Figure 5C). These data strongly suggest that HSV-2 and TLR-3 agonist Poly(I:C) affect LC function and thereby increase HIV-1 transmission to T cells.

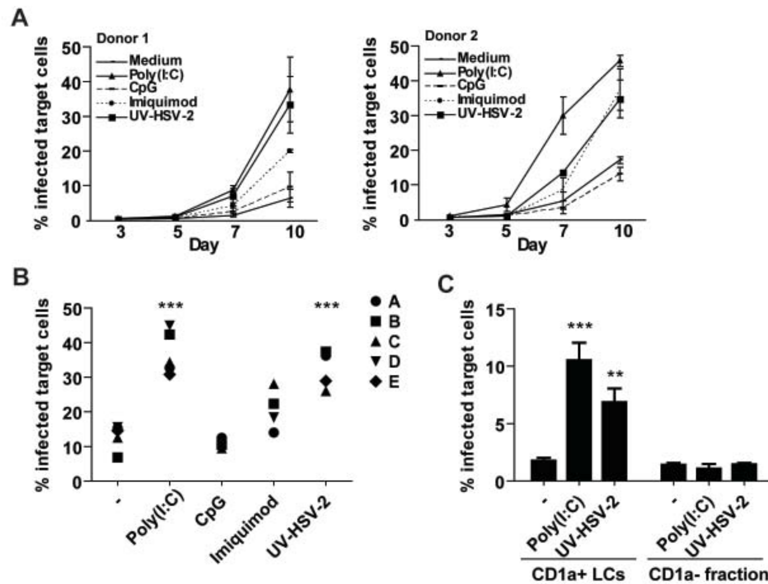

Figure 5. Both HSV-2 and Poly(I:C) stimulation of LCs enhances HIV-1 transmission. (A,B) Epidermal LC fraction was stimulated for 18 hrs. Cells were washed extensively and incubated with R5-tropic HIV-1-eGFP for 2 hrs. Cells were washed and CCR5+ Jurkat cells were added. Transmission of HIV-1 was followed over time by flow cytometry. (A) Time course of the transmission of HIV-1 for two different donors. (B) Combined results for five donors are depicted. (C) CD1a+ LCs and CD1a- fraction were stimulated for 18 hrs with Poly(I:C) or UV-HSV-2. Cells were washed and incubated with R5-tropic HIV-1-eGFP for 40 hrs. CCR5+ Jurkat cells were added and transmission of HIV-1 was followed over time by flow cytometry. Results are representative for two independent donors. Error bars represent standard deviations of duplicates.* p<0.05, ** p<0.01, *** p<0.001.

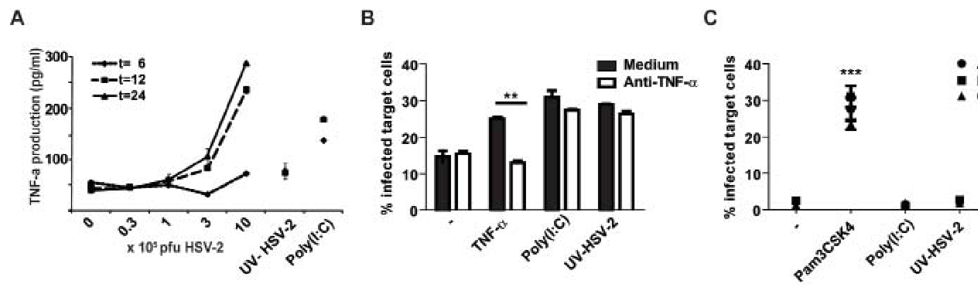

Figure 6. Direct infection of LCs but not TNF-α production and *trans*-infection contribute to enhanced HIV-1 transmission by HSV-2 and Poly(I:C). (A) Epidermal LC fraction was incubated with different concentrations HSV-2 for 6, 12 and 24 hrs with HSV-2, UV-HSV-2 or Poly(I:C). Supernatants were harvested and TNF-α protein production was measured by ELISA. Error bars represent standard deviations of duplicates. These results were observed for three donors. (B) Epidermal LC fraction was stimulated for 18 hrs. Cells were washed extensively and pre-incubated with anti-TNF-α followed by an incubating with R5-tropic HIV-1-eGFP for 2 hrs. CCR5+ Jurkat cells were added and transmission of HIV-1 was followed over time by flow cytometry. Addition of anti-TNF-α did not alter the enhanced transmission by HSV-2 and Poly(I:C) stimulated LCs. Error bars represent standard deviations of duplicates. (C) Replication-defective R5-tropic HIV-1-eGFP was used in the transmission assay. HSV-2 and Poly(I:C) do not transmit HIV-1 via *trans*-infection, whereas Pam3CSK4 does. Error bars represent standard deviations of duplicates. These results were observed for three donors. * p<0.05, ** p<0.01, *** p< 0.001.

TNF-α production and *trans*-infection do not determine the enhanced HIV-1 transmission by HSV-2 and Poly(I:C)

The pro-inflammatory cytokine TNF-α enhances HIV-1 replication (16;24;25). Therefore, we investigated whether TNF-α production was involved in the enhanced HIV-1 infection of LCs after HSV-2 and Poly(I:C) incubation. Epidermal LC fraction was stimulated with HSV-2, UV-HSV-2 or Poly(I:C) and the production of TNF-α was measured at different time points. HSV-2 and UV-HSV-2 produced low levels of TNF-α, which increased over time and was concentration dependent. Poly(I:C) also induced low levels of TNF-α production (Figure 6A). To investigate whether TNF-α production induced the enhanced HIV-1 transmission by HSV-2 and Poly(I:C), a neutralizing antibody against TNF-α was added during the transmission assay (16). Anti-TNF-α antibody did not significantly affect HIV-1 transmission induced by UV-HSV-2 and Poly(I:C), whereas it did abrogate TNF-α-induced HIV-1 transmission (Figure 6B). These data show that TNF-α is not responsible for the observed enhanced HIV-1 transmission by LCs after Poly(I:C) or HSV-2 stimulation.

Our data strongly suggest that HSV-2 and TLR3 ligands increase transmission of HIV-1 by *de novo* production of HIV-1 by LCs. Previously it has been demonstrated that Pam3CSK4-activated LCs (16) and LPS/TNF-α activated LC-like cells (26) enhance HIV-1 transmission without LC infection, a mechanism known as *trans*-infection. Although we observed an enhanced replication of HIV-1 in LCs in response to HSV-2 and Poly(I:C) (Figure 3B), we investigated whether HSV-2 or Poly(I:C) could enhance HIV-1 *trans*-infection. Replication-defective R5-tropic pseudotyped HIV-1ΔENV-eGFP was used to monitor HIV-1 trans-infection. In contrast to Pam3CSK4, both HSV-2 and Poly(I:C) did not transmit replication-defective HIV-1 to T cells (Figure 6C). These data suggest that HSV-2 and TLR-3 agonist Poly(I:C) enhance HIV-1 transmission by increasing HIV-1 infection of LCs.

Discussion

Genital HSV-2 infection is highly prevalent in sexually active people world-wide, with a prevalence in the general population of around 20% in the USA and 60% in sub-Saharan Africa (27). Epidemiological studies have demonstrated that HSV-2 infection increases the risk of HIV-1 acquisition even in the absence of clinical symptoms (1-5). Here we have demonstrated that HSV-2 strongly increases HIV-1 transmission by LCs. Immature LCs form a protective barrier against HIV-1 infection through of the C-type lectin Langerin (15;20-22). However, our data suggest that HSV-2 interferes with this anti-HIV-1 function by decreasing Langerin expression and saturating Langerin activity. This results in increased HIV-1 infection of LCs and subsequent transmission to T cells. These data show that, aside from the previously described mechanisms, viral STIs also enhance HIV-1 susceptibility by altering LC function.

Although epithelial cells and keratinocytes are the primary target cells for HSV-2, we demonstrate that LCs are productively infected by HSV-2. HSV-2 infection of LCs induced a mature phenotype with an increased expression of co-stimulatory molecules but a decreased expression of Langerin, whereas expression of CD4

and CCR5 was not affected. Immune activation by HSV-2 was independent of viral replication since we observed a similar maturation with UV-inactivated HSV-2. HSV-2 has been shown to interact with TLR-2, which is expressed by LCs and might be involved in LC maturation (28). We have previously demonstrated using an *ex vivo* model that TLR-1/-2 ligand Pam3CSK4 did not affect Langerin expression on LCs (16). This is in contrast with HSV-2, which induced maturation of LCs and these data suggest that HSV-2 triggers other receptors as well that might be involved in LC maturation.

Comparison between different viral TLR agonists showed that TLR-3 agonist Poly(I:C) was the most potent in inducing LC maturation as well as decreasing Langerin expression, whereas TLR-7/-8 agonist Imiquimod had a marginal effect on LC maturation and Langerin expression. As expected TLR-9 agonist CpG did not affect LC function since TLR-9 is not expressed by LCs (19). These data strongly suggest that HSV-2 as well as other viruses containing TLR-3 ligands induce LC maturation and affect Langerin expression.

Notably, our data show that HSV-2 also interacted with Langerin. These data support a function for Langerin as a pattern recognition receptor (29) that interacts with different pathogens including viruses such as HIV-1, HSV-1 (data not shown) and HSV-2, *Mycobacterium tuberculosis* and *Candida albicans* (15;20-22). The function of Langerin as a pattern recognition receptor is unclear. HSV binding to Langerin might result in more efficient antigen processing and presentation (21;30). Previously we have shown that Langerin prevents HIV-1 infection of LCs by capturing HIV-1 for degradation (15). Thus, Langerin might perform a similar function for HSV-2. However, LCs express high levels of proteoglycans such as syndecan-3 (31), that function as an attachment receptor for HSV-2 and might facilitate HSV-2 infection even in the presence of Langerin. Further studies will be necessary to identify the function of Langerin in HSV infection.

Langerin is an important C-type lectin on LCs that prevents HIV-1 infection by binding and degrading HIV-1(15;32) Previous studies have shown that inhibition of Langerin by a blocking antibody or a carbohydrate ligand increases HIV-1 infection of LCs (15). Notably, our data show that both HSV-2 and TLR-3 agonist Poly(I:C) decreased Langerin expression, which prevented efficient HIV-1 binding by LCs. Moreover, HSV-2 competed with HIV-1 for Langerin binding thereby further hampering the protective function of Langerin. Thus, HSV-2 affects Langerin function at different levels and this prevents HIV-1 capture, leading to increased HIV-1 infection of LCs and subsequent transmission to T cells. Various studies have shown that HSV can increase HIV-1 replication at several levels (33-35) and this might also affect HIV-1 infection of LCs. However, our data show that HSV-2 infection is not required, since UV-inactivated HSV-2 induced HIV-1 infection and transmission to a similar level as replication-competent HSV-2. These data suggest that aside from competition of HSV-2 with HIV-1 for Langerin binding, innate signaling by pattern recognition receptors induces LC maturation and this might also affect HIV-1 replication. Similarly, we cannot exclude that TLR-3 triggering by Poly(I:C) also affected HIV-1 replication by activating NF-κB and other transcription factors that might exert an

additive effect on HIV-1 replication.

We have previously demonstrated that TLR-1/-2 ligand Pam3CSK4 enhanced HIV-1 transmission by LCs via trans-infection (16). HSV-2 has been shown to interact with TLR-2 but notably HSV-2 enhanced HIV-1 infection of LCs, suggesting that it activates LCs through a different mechanism. The differences observed in maturation status might account for the differences in HIV-1 susceptibility.

A recent study showed that TLR-3 ligand Poly(I:C) does not enhance HIV-1 transmission (36), whereas our data show that Poly(I:C) is a potent inducer of LC maturation and HIV-1 transmission. The differences might be due to the model used, since they obtained LCs with a mature phenotype after migration from tissues which complicates analyses of effects on LC maturation. Thus, both Poly(I:C) and HSV-2 increased HIV-1 infection of LCs by affecting Langerin function but whether innate signaling also affect HIV-1 replication needs to be further elucidated.

Immune activation is an important determinant in HIV-1 replication and infection. Pro-inflammatory cytokines such as TNF-α and IL-1β enhance HIV-1 replication (16;24;25). We have previously demonstrated that fungal pathogens induce the production of TNF-α in mucosal tissue which increases HIV-1 transmission by LCs (16). However, HSV-2 infection induced low TNF-α and no IL-1β production (data not shown) and, in addition, anti-TNF-α did not abrogate the enhanced HIV-1 transmission observed in the presence of HSV-2 or Poly(I:C). These data strongly suggest that TNF-α is not involved in the HSV-2- or Poly(I:C)-enhanced HIV-1 infection of LCs. Previously we have shown that TNF-α and TLR-2 agonist Pam3CSK4 increase HIV-1 transmission by enhancing HIV-1 replication and HIV-1 capture, respectively (16). Here we have identified another mechanism that affects HIV-1 infection of LCs: viral pathogens or viral TLR agonists hamper Langerin expression and function, which increases HIV-1 infection of LCs (15;16;26). A recent study has demonstrated that immune infiltrates persist well after the HSV-2 lesion has healed. The increased expression of DC-SIGN on DCs in these infiltrates and T cell influx might contribute to HIV-1 susceptibility (10). Further studies will be necessary to investigate the expression of Langerin on LCs present in the healed HSV-2 lesions since prolonged immune activation might lead to decrease antiviral function of LCs.

Collectively our data supports a role for LCs in the enhanced susceptibility to HIV-1 in the presence of HSV-2. HSV-2 infection abrogates the anti-HIV-1 function of LCs by inducing LC activation, and decreasing Langerin expression and function. Continuous HSV-2 shedding by mucosal tissue will maintain the presence of HSV-2, which will further block Langerin function and induce activation of LCs. Furthermore, double-stranded RNA viruses that are mimicked by Poly(I:C) might also increase HIV-1 susceptibility. Our data emphasize the importance of STI prevention, since they form a risk factor to acquire HIV-1, even in the absence of clinical symptoms.

Materials and Methods

Antibodies and reagents. PE-conjugated antibodies against Langerin, CD80, CD86, CCR5 and CD4 (BD Pharmingen), CD1a-FITC (BD Pharmingen), CD1a-PE (Abcam), HSV1/2 gB (Novus Biologicals), goat anti-mouse-FITC (Zymed Laboratories Inc.). TLR agonists Poly(I:C) (10 µg/ml, Sigma-Aldrich), Imiquimod (10 µg/ml, Invivogen), CpG ODN 2216 (10 µg/ml, Invivogen), and Pam3CSK4 (5 µg/ml, Invivogen). Recombinant human TNF-α (0.1 µg/ml, Strathmann biotec), anti-TNF-α (20 µg/ml, Biovision).

Cell lines and viruses. Jurkat T cells expressing CCR5 were generated by retroviral transduction as previously described (37;38). The HSV-1 strains Syn17^{+} and HSV-2 strain 333 were grown on green monkey kidney (GMK) cells and harvested after 24 hours of infection. Supernatant was filtrated over a 0.22 µm filter and a plaque titration assay was performed to determine viral titres (39). HSV-2 was inactivated by placing a Petri-dish containing 3 ml of viral suspension under a UV- lamp for 10 min on ice. Virus inactivation was confirmed and the virus stocks were stored at -80°C until further use. Medium from cultured GMK cells was treated similarly and used as a mock control.

HIV-1 viruses R5-tropic eGFP-expressing HIV-1-NL4.3-BaL (referred to as HIV-1-eGFP) (20ng/well) and replication-defective R5-tropic pseudotyped HIV-1 (HIV-1 BaL-pseudotyped NL4.3-eGFPΔENV) (50ng/well) were produced in 293T cells as described before (16). Virus stocks were quantified by p24 ELISA (Perkin Elmer Life Sciences) and titrated using the indicator cells TZM-blue (16;40).

Isolation of epidermal single cell suspension and LCs. Human tissue was obtained from healthy donors undergoing corrective breast or abdominal surgery after informed consent in accordance with institutional guidelines and used within 3 hrs after surgery. An LC-enriched epidermal single cell suspension was generated as described previously (15;16). In short, epidermis was separated after dispase II (1 mg/ml) (Roche Diagnostics) treatment, and digested in 0.05% trypsin solution. Cell suspension was purified over Lymphoprep gradient. As determined by CD1a/ Langerin staining, these single cell suspensions generally contained 10-15% LCs. CD1a^{+} LCs were isolated using positive selection for CD1a in magnetic-activating cell sorting (MACS) according to manufacturer's protocol (Miltenyi biotec). This yielded a >90% pure CD1a^{+} LC population. Cells were cultured in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10% FCS and 10µg/ml gentamycin.

Cell stimulation. Cells (1x10^{5}) were pre-incubated with TLR stimuli, HSV-2 (1x 10^{5} p.f.u.), UV-HSV-2 (1x 10^{5} p.f.u.) or a mock control overnight. Flow cytometric analysis for cell surface markers was performed. To determine productive HSV-2 infection of LCs, 1x10^{5} cells were incubated with different viral input and expression of HSV-2 glycoprotein B (gB) was measured by flow cytometry. A double staining for CD1a was performed to analyse infection of LCs. For TNF-α production cells were incubated with different concentrations HSV-2 or UV-HSV-2 for 6, 12 and 24 hrs or with Poly(I:C). Supernatant was harvested and TNF-α protein production was measured by ELISA (Biosource).

Fluorescent bead adhesion assay. Strepavidin-coated beads (TransFluorSpheres, Molecular Probes) were coated with HIV-1 gp120 as described previously (41). The adhesion assay was performed as follows (39;42;43): 1x 10^{5} cells were incubated overnight with TLR agonists or different concentrations of HSV-2 and UV-HSV-2. Cells were washed extensively with PBS and stained with CD1a-FITC. Next, cells were washed in TSA buffer (Tris buffer (20 mM Tris-HCL, pH 7, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) (TSM) supplemented with 0.5% BSA) before adding HIV-1 gp120 beads for 45 min at 37°C. Cells were washed and binding was measured by flow cytometry.

Langerin binding Elisa. Different concentrations of HSV-2 were coated onto ELISA plates overnight at room temperature. Non-specific binding was blocked by incubating the plate with TSA buffer for 1 h at 37°C. Recombinant human Langerin (23) (2 µg/ml) was added for 1 h at 37°C. Unbound Langerin

was washed away and binding was determined using an anti-Langerin antibody (DCGM4, Beckman Coulter Inc) followed by peroxidise-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch). Specificity was determined in the presence of mannan (1 mg /ml) or EGTA (10 mM/ml).

HSV-2 mRNA transcription. 1x 10^5 $CD1a^+$ LCs (>95% pure) were infected with different concentrations HSV-2 or UV-HSV-2. After 6, 24 or 48 hrs the cells were washed extensively with PBS and mRNA was isolated with the mRNA capture kit (Roche). cDNA was synthesized with the Reverse transcriptase kit (Promega). For quantitative real-time PCR analysis, PCR amplification was performed in the presence of SYBR green, as previously described (16;44). Specific primers for HSV-2 thymidine kinase and GAPDH were designed by Primer Express 2.0 (Applied Biosystems). Forward primer sequence: CGAGCCGATGACTTACTGGC reverse primer sequence: GCTGCGTGTTGTAGATGTTCG. Transcription was adjusted for *GAPDH* transcription.

Immunofluorescence microscopy. $CD1a^+$ LCs ($1x10^5$; >95% pure) were incubated with $1x10^5$ p.f.u. HSV-2 or UV-HSV2 for 24 hrs. Cells were washed and stained intracellular for HSV2-ICP27 or HSV-2 ICP5 in combination with CD1a (all Santa Cruz Biotechnology). Cells were analyzed by confocal microscopy (Leica AOBS SP2 CSLM system).

HIV-1 infection. Overnight stimulated cells were washed extensively and inoculated with R5-tropic HIV-1-NL4.3-BaL (20ng/well). For mRNA analysis, after 6 and 24 hrs cells were washed extensively with PBS and host and viral mRNA were isolated with the mRNA capture kit (Roche). For real-time PCR analysis, PCR amplification was performed in the presence of SYBR green, as previously described (16;44). HIV-1 Tat/Rev transcripts were measured by using specific primers which detect both *Tat* and *Rev* mRNA across an intron (sequence see (16)). Transcription was adjusted for *GAPDH* transcription and relative mRNA expression of HIV-1-infected control samples was set at 1.

HIV-1 transmission. Cells were washed extensively and inoculated with HIV-1-eGFP (20ng/well) or replication-defective R5-tropic pseudotyped HIV-1-eGFPΔENV (50ng/well). After 2 and 40 hrs, cells were washed and $CCR5^+$ Jurkat T cells ($2x10^4$) were added. Transmission to $CCR5^+$ Jurkat T cells was measured over time by flow cytometry.

Statistical analysis. To compare the means of multiple groups, a one-way analysis of variance (ANOVA) was used. When the overall F-test was significant, differences were investigated further using a post-hoc Bonferroni test using Graphpad Prism software. *P* value of <0.05 was considered statistically significant. * p<0.05, ** p<0.01, *** p<0.001.

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