Chapter 6.4

Herpes simplex virus enhances HIV-1 transmission by Langerhans cells.


Genital herpes is a risk factor to acquire HIV-1 infection by sexual contact, by increasing both infectivity of an infected person and susceptibility to acquire HIV-1. Here we have investigated the mechanisms that underlie the increased susceptibility to HIV-1 when people have genital herpes. Under steady state conditions, LCs capture HIV-1 through interaction with the C-type lectin Langerin, resulting in viral clearance and protection against HIV-1. During primary genital herpes or recurrent infections, HSV infects epithelial cells and encounters LCs in the mucosal epithelium. We set out to investigate the function of HSV-infected LCs and subepithelial DCs during HIV-1 transmission. We demonstrate that HSV interacts with soluble and cellular Langerin and that infection of LCs decreases expression of this receptor. Notably, LC infection with both HSV-1 and HSV-2 enhances HIV-1 transmission, suggesting an important role for LCs during HIV-1 transmission in the HSV-infected individual. Furthermore our data indicate that HSV infection of DCs and LCs has different outcomes for HIV-1 transmission, and our results suggest that DC-SIGN and Langerin are involved. Future research is needed to confirm the differential role of Langerin and DC-SIGN during HIV-1 transmission in conditions of genital herpes.

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Introduction
Genital herpes is a common infection, which is mainly caused by herpes simplex virus type-2 (HSV-2), although an increasing percentage of the genital herpes is caused by HSV-1. Genital herpes is a risk factor to acquire HIV-1 infection by sexual contact, by increasing both infectivity and susceptibility to acquire HIV-1. In this chapter, we have investigated the mechanisms that underlie the increased susceptibility to HIV-1 when people have genital herpes.

Genital herpes is characterized by the formation of papules and vesicles, which can progress into pustules and ulcers. Ulceration disrupts the mucosal barrier and thereby abrogated the protective barrier function of the epithelium and the clearance of HIV-1 virions by Langerhans cells (LCs). Moreover, ulceration allows HIV-1 to reach the subepithelial dendritic cells (DCs), which efficiently promote HIV-1 transmission in vitro. Furthermore, target cells for HIV-1 are attracted to the mucosal sites during HSV-2 infection, which can result in higher transmission rates. Since LCs and subepithelial DCs are thought to be crucial during the sexual transmission of HIV-1, we investigated whether HSV infection of LCs and subepithelial DCs alters their function and promotes HIV-1 transmission.

In normal situations, LCs capture HIV-1 through interaction with the C-type lectin Langerin, resulting in viral clearance and protection against HIV-1. Blocking Langerin results in LC infection and transmission of HIV-1 to T cells. During primary genital herpes or recurrent infections, HSV infects epithelial cells and encounters LCs in the mucosal epithelium. HSV interaction with LCs might alter the protective function of Langerin or increases susceptibility to HIV-1 by upregulating other factors, such as expression of CD4 and CCR5. DC-SIGN⁺ DCs effectively transmit HIV-1 to target cells in vitro.

Genital HSV infection attracts DC-SIGN⁺ DCs towards the site of infection, and ulceration will result in interaction of HIV-1 with subepithelial DC-SIGN⁺ DCs. HSV can infect the DC-SIGN⁺ DCs leading to DC activation or induction of maturation of the cells through interaction with Toll-like receptor ligands. Activation of DCs has previously been demonstrated to enhance transmission of HIV-1, and we therefore investigate whether HSV-infected DCs enhance HIV-1 transmission.

Here we set out to investigate the function of HSV-infected LCs and subepithelial DCs during HIV-1 transmission. We demonstrate that HSV interacts with both soluble and cellular Langerin. HSV-1 and -2 decrease Langerin expression on LCs and enhances transmission of HIV-1 by LCs. In conclusion our data propose an important role for LCs during HIV-1 transmission in the HSV-infected individual.

Results
Langerhans cells are not efficiently infected by HSV
DC-SIGN and heparan sulfates capture HSV and enhance infection of moDCs, a model for subepithelial DC-SIGN⁺ DCs. However, genital herpes is an epithelial infection and therefore HSV first encounters LCs residing in the epithelium. Little is known about the infection of human LCs with HSV. Bosnjak et al. refer to their unpublished data that LCs are infected by HSV. To investigate the susceptibility of LCs to HSV-1, we infected moDCs and emigrant primary human LCs with different concentrations of HSV-1 and analyzed the expression of HSV gB on the cell surface to determine infection. In contrast to moDCs, emigrant LCs did not express detectable levels of gB, even at higher inoculums of HSV and after 24/48 hours (Figure 1a). In some donors we observed some infection at higher viral input (data not shown), however levels were always low compared to moDCs. These results demonstrate that LCs can be infected with HSV, albeit not efficiently. Preliminary data demonstrate infection of LCs with HSV-2 is also inefficient compared to moDCs (personal communications Marein de Jong).
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Figure 1. Emigrant Langerhans cells not efficiently infected with herpes simplex virus type-1.
(a) moDCs and emigrant LCs were infected with different concentration of HSV-1. After 24 hours the cells were harvested and stained for the cell surface expression of HSV gB and analyzed by flow cytometry. Infection is depicted as mean fluorescent intensity of the staining for gB. Error bars represent the standard error of the mean of two donors. (b) moDCs and emigrant LCs were analyzed for the expression of syndecan 1-4 and heparan sulfates by flow cytometry. Unfilled histograms represents isotype control and filled histograms antibody specific staining. Error bars represent the standard error of the mean of two donors.

Different factors are involved in HSV infection, such as the expression of entry receptors, attachment receptors and possibly other restrictive factors. It has been described that heparan sulfates play an important role in the attachment of HSV to target cells. We therefore investigated the expression of heparan sulfates and heparan sulfate proteoglycans (HSPG) on emigrant LCs. As shown in Chapter 4.2, emigrant LCs express low levels of heparan sulfates and syndecan-3, a HSPG abundantly expressed by moDCs. Similar to moDCs, LCs do not express high levels of syndecan-1, -2 and -4 (Figure 1b). Thus, low levels of heparan sulfates can be responsible for the low susceptibility of LCs to HSV infection.

Langerin is a receptor for HSV

High mannose structures are ligands for both Langerin and DC-SIGN, and therefore both C-type lectins bind HIV-1 gp120. DC-SIGN is a receptor for both HSV-1 and HSV-2 (Chapter 4), however the interaction of these viruses with Langerin is not known. We investigated whether HSV interacts with Langerin using a soluble Langerin binding ELISA. HSV particles were coated and binding of soluble Langerin was measured by ELISA. Langerin interacts with both HSV-1 and HSV-2 (Figure 2a). The binding was specific for the calcium dependent carbohydrate recognition domain of Langerin, since it could be blocked by the polycarbohydrate mannan and the calcium-chelator EGTA (Figure 2a).
As previously demonstrated\(^{18}\), HIV-1 gp120 is a ligand for Langerin, and here we demonstrate that Langerin binds to recombinant gp120 of both R5- and X4-using viruses (Figure 2a). Next, we investigated whether cellular Langerin is a receptor for HSV using the HIV-1 gp120 bead adhesion competition assay\(^{56}\). Parental Raji and Raji-Langerin transfectants were incubated with HSV-2 and subsequently the binding to HIV-1 gp120 beads was measured. As previously demonstrated, HIV-1 gp120 interacts with Raji-Langerin cells\(^{18}\), but not with parental cells (Figure 2b). The interaction is mediated by the carbohydrate recognition domain of Langerin, since blocking the receptor with mannan or EGTA abrogated the binding to levels observed with the parental cells. Pre-incubation of the Raji-Langerin cells with HSV-2 reduced the binding of HIV-1 gp120 beads, indicating that HSV-2 competes for binding with HIV-1 gp120 and that HSV-2 interacts with Langerin (Figure 1b). Thus, Langerin is a receptor for HSV-2. The interaction of HSV-1 and cellular Langerin is under current investigation.

**Figure 2. Langerin is a receptor for herpes simplex virus.**
(a) Binding of soluble Langerin to HSV-1, HSV-2, recombinant gp120 of R5 using HIV-1 BaL and CM235 and X4 using HIV-1 IIIB was measured using the soluble Langerin ELISA. The recombinant Langerin was pre-incubated with mannan or EGTA to determine the specificity of the interaction. Error bars represent the standard deviation of the duplicates in one experiment. A representative experiment of three is depicted. (b) Binding of HSV-2 to Langerin was measured using the HIV-1 gp120 beads competition assay. Raji or Raji-Langerin cells were pre-incubated with mannan, EGTA, anti-Langerin or HSV-2 and binding of HIV-1 gp120 beads was measured by flow cytometry. Error bars represent the standard error of the mean of the duplicates in one experiment. A representative experiment of two is depicted.

**HSV-1 and -2 decrease expression of Langerin and enhance HIV-1 transmission by LCs to T cells.**
The C-type lectins, Langerin and DC-SIGN are key players in HIV-1 transmission\(^{18}\). Binding of HIV-1 to DC-SIGN mediates HIV-1 transmission, whereas Langerin binds HIV-1 and prevents LC infection and subsequent transmission. We have shown that Langerin and DC-SIGN are both receptors for HSV, and there is epidemiological evidence that HSV co-infection increases the susceptibility to acquire HIV-\(^{188}\). These facts prompted us to investigate whether the interaction of HIV-1 with HSV-infected DCs/LCs attributes to enhance HIV-1 transmission during genital herpes. Langerin and DC-SIGN expression were analyzed after HSV infection. Interestingly, both Langerin and DC-SIGN cell surface expression are decreased after LCs respectively DC infection by HSV-1 and -2 (Figure 3a), suggesting that the function of both C-type lectins is altered after HSV infection.

Next, HSV-infected LCs were incubated with HIV-1, washed and transmission to T cells was measured by p24 ELISA. LCs do not efficiently mediate HIV-1 transmission, however, mannan pre-treatment enhances HIV-1 transmission (Figure 3b). This was previously demonstrated to be due to blocking Langerin function\(^{18}\). Strikingly, HSV-infection of LCs enhances HIV-1 transmission. Moreover,
transmission levels were enhanced to the same levels as mannan pre-treatment. Thus, both HSV-1 and HSV-2 decrease Langerin expression and enhance HIV-1 transmission, suggesting that HSV inhibits HIV-1 degradation by Langerin, and allow infection of LCs via CD4/CCR5 and subsequent transmission to T cells. DCs efficiently transmit HIV-1 and this infection partially mediated by DC-SIGN (Chapter 2). Our results indicate that DC infection with HSV-1 and to a lesser infection with HSV-2 decrease HIV-1 transmission, suggesting that HSV-infected DCs are less able to mediate HIV-1 transmission, and therefore are not involved in the enhanced transmission in vivo (data not shown).

Figure 3. Herpes simplex infection decreases Langerin expression and enhances transmission of HIV-1 by Langerhans cells. (a,b) moDCs and emigrant LCs were infected with HSV-1 and HSV-2 (MOI 1) overnight. (a) The cells were analyzed for Langerin expression. Unfilled histograms represent isotype control and filled histograms antibody specific staining. The mean of the specific staining is depicted (b) Uninfected LCs were pre-treated or not with mannan. Uninfected and HSV-infected cells were incubated with HIV-1 JRCSF for 2 hours. The cells were washed extensively and incubated with activated CD4+ T cells. At different days supernatants were collected and HIV-1 infection was determined by p24 ELISA. Error bars represent the standard deviations of the triplicates in one experiment. A representative experiment of two is depicted.
Discussion

Here we have investigated the interaction of HSV and LCs and the role of HSV-infected LCs for HIV-1 transmission. We have demonstrated that HSV interacts with soluble and cellular Langerin and that infection of LCs decreases expression of this receptor. Notably, LC infection with both HSV-1 and HSV-2 enhances HIV-1 transmission. These results strongly suggest a role for HSV-infected LCs in the enhanced transmission of HIV-1 during genital herpes. Little has been reported on the infection human LCs with HSV. In mice, vaginal and cutaneous LCs are not responsible for virus-specific CD8+ and CD4+ T cell priming3,117. However, LCs are thought to be important during HSV infection, since depletion from mouse skin enhances peripheral HSV infection96. Here we demonstrated that human emigrant LCs are barely infected with HSV-1, in contrast to moDCs. Since moDCs are a model system for dermal DCs, there may be differences between these cell-types. To address this question we are currently investigating infection levels of immature LCs compared to immature dermal DCs. Ex vivo infection of epidermal skin sheets demonstrates that keratinocytes are efficiently infected, in contrast to epidermal immature LCs (data not shown), supporting our data that LCs are not efficiently infected by HSV. This could have implications for understanding the results in the murine models, since infection of DC/LCs, rather than cross-presentation, might play a role in presentation of antigens on MHC class-I and subsequent T cell priming. This is even more important since cross-presentation of viruses or virus-infected cells by LCs is inefficient (Chapter 5.3).

Langerin captures HIV-118 and MV and hereby prevents infection of LCs through CD4/CCR5 and SLAM, respectively. Since we have demonstrated here that Langerin is a receptor for HSV-1 and -2, we investigated whether a similar mechanism is involved for LC infection with HSV. Interestingly, mannan could not confer susceptibility to levels seen for moDCs (data not shown), indicating that other factors restrict LC infection by HSV, such as the expression levels of the entry or attachment receptors for HSV. The expression of heparan sulfates are lower on emigrant LCs compared to moDCs, which could result in reduced attachment of virus and subsequent infection, however high viral inoculums did not overcome low attachment, suggesting other restriction factors on the level of entry, fusion or replication. Infection of LCs and DCs with HSV decreases expression of Langerin and DC-SIGN. This could be a result of receptor-mediated endocytosis and subsequent loss of cell-surface expression32 of the receptors or due to DC maturation and subsequent down-regulation of de novo synthesis of the receptor. Notably, moDC activation upon HSV infection is atypical, including down-regulation of co-stimulatory molecules but not MHC-I and -II69. Here we report that DC-SIGN is down-regulated upon HSV infection, which coincides with TLR-mediated DC maturation34.

Since, genital herpes is a risk factor to acquire HIV-188, and HSV infection affects expression of the HIV-1 receptors Langerin and DC-SIGN18, we investigated the role of HSV-infected DC subsets in HIV-1 transmission. Interestingly, our data indicate that HSV-infected DCs transmitted HIV-1 less efficient. This might be due to lower expression of DC-SIGN, competitive interaction of both viruses for this receptor, or apoptosis of DCs9. Moreover, decreased expression of co-stimulatory molecules69 might affect the formation of the virological synapse67, and subsequent transfer of virus. TLR stimuli, such as Poly I:C, enhance DC mediated HIV-1 transmission90. These data stress that activation of DCs by HSV is distinct to TLR-mediated maturation, with regard to HIV-1 transmission. Thus, DC-SIGN+ DCs are not involved in the increased transmission during genital herpes.

In contrast, HSV-1 and -2-infected LCs enhanced transmission of HIV-1 to T cells to similar levels as pre-treatment with mannan. This suggests that HSV infection affects Langerin function on LCs. Indeed, Langerin expression is down-regulated upon infection and furthermore free HSV particles might compete for HIV binding to residual Langerin. During HSV infection of the genital area there is a complex interplay between HSV target cells (epithelial cells) and Langerhans cells. Here we show that
HSV can interact directly with Langerhans cells, thereby down-regulating Langerin expression and increasing HIV-1 transmission. However, we cannot exclude that the environment created by HSV infected epithelial cell will also modulate Langerin function on Langerhans cells. Since we observe increased transmission of HIV-1 by HSV-infected LCs, these two mechanisms can probably exist next to each other and do not exclude each other. Abrogated Langerin function decreases HIV-1 capture by Langerin and subsequent degradation, and allows infection of LCs through CD4/CCR5 and subsequent transmission to T cells. Other mechanisms could also have been involved, such as increased expression of CD4 and CCR5, or increased capture and infection independent transmission as seen after PAM3CSK4 interaction (Chapter 6.3). In chapter 6.3 we have demonstrated that TNFα enhances the replication of HIV-1 in LCs and subsequent transmission to T cells, which might play a role during genital inflammation Preliminary data have demonstrated that HSV infection of epidermal sheets and skin biopsies does not induce TNFα expression (data not shown). Therefore, the mechanism described in chapter 6.3 is not thought to play a role in enhanced HIV-1 transmission during genital herpes.

Together these data indicate that HSV infection of DCs and LCs has different outcomes for HIV-1 transmission, and our results suggest that DC-SIGN and Langerin are involved. We can, however, not exclude that other factors have attributed to this observation, such as the different susceptibility to HSV of moDCs and emigrant LCs and subsequent apoptosis, the comparison between a immature DC-model and primary mature cells. Future research is needed to confirm the differential role of Langerin and DC-SIGN during genital herpes.

As the HIV/AIDS pandemic is still an increasing burden, novel control measures for the spread of HIV are urgently needed. Here we demonstrate that HSV-infected LCs express lower levels of Langerin and are more prone to mediate HIV-1 transmission, indicating an important function of this DC subset during HSV infections. These results stress the importance to treat and prevent genital herpes. Moreover, topical microbicides in a herpes simplex virus setting should aim at preserving Langerin function and decreasing LC infection.

**Materials and Methods**

**Antibodies, cell lines, proteins and viruses.** The following antibodies were used: mouse antibodies against DC-SIGN AZN-D133.34, mouse antibodies against Langerin 10E218, HSV specific mouse antibody against HSV gB T111 (Novus biologicals, Littleton, CO, USA), mouse monoclonal antibodies against syndecan-1 B-B4, syndecan-2 10H4, syndecan-3 1C7, syndecan-4 8G3 and monoclonal antibodies 10E4 and 3G10, recognizing heparan sulfate chains and heparinase digested heparan sulfate epitopes, respectively115 (a generous gift of Dr. G. Davids), PE-labeled and unlabelled DCGM4 (Beckman Coulter Inc., Miami, Florida, USA), goat anti-human IgG conjugated with PO (Jackson Immunoresearch, West Grove, PA, USA), goat anti-mouse IgG (H’L) antibody conjugated with FITC (Zymed Laboratories Inc., South San Franciso, Ca. USA). Culture and generation of stable Raji transfectants expressing Langerin was previously described18. The HSV-1 virus strain Syn17+ and HSV-2 strain 333 were grown and titrated on Green Monkey Kidney (GMK) cells. The HIV-1 virus strain was grown and titrated on activated CD4+ T cells. The following proteins were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 IIIB gp120, HIV-1 BaL gp120, and HIV-1CM235 gp120.

**Primary cell culture assay.** Immature monocyte derived DCs (moDCs) were cultured as described before (Sallusto & Lanzavecchia, 1994). In short, human blood monocytes were isolated from buffy coats by use of a Ficoll gradient and a subsequent CD14 selection step using a MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Purified monocytes were differentiated into immature DCs in the presence of interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF), respectively 500 and 800 U/ml (Schering-Plough, Brussels, Belgium). Experiments using immature DCs were performed at between day 5 and 7 in RPMI containing 10% FCS, IL-4 and GM-CSF. Primary emigrant Langerhans cells were prepared from normal healthy
skin obtained from plastic surgery as described before. In short, a dermatome was used to cut 3mm thick slices containing both dermis and epidermis within three hours after surgery. Dermal and epidermal tissue was separated mechanically after 1mg/ml Dispase II (Roche Diagnostics, Penzberg, Germany) treatment in Iscoves Modified Dulbecco’s Medium (IMDM), 10% FCS and 10µg/ml gentamycine for either 2 hours at 37°C or 18 hours at 4°C. Epidermal sheets were cultured in IMDM with 10% FCS, 10µg/ml gentamycine, 10 U/ml penicilline and 10 µg/ml streptomycin. After 3 days, epidermal sheets were removed and cells were layered on a Ficoll gradient, and cultured at 5x10^5 /ml in IMDM, 10% FCS, 10µg/ml gentamycine and 500U/ml interleukin-4 (IL-4) and 800U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF).

**HSV-1/-2 infection.** Immature DCs and emigrant LCs were seeded in a round bottom 96 wells plate (Greiner Bio-One, Frickenhausen Germany) and infected with different concentrations of HSV-1 or HSV-2. After 24 hours the cells were harvested and stained with antibodies against HSV gB (T111). The cells were washed and fixed with 4% PFA/PBS and analyzed by flow cytometry.

**Soluble langerin binding assay.** HSV-1 and -2 were centrifuged at 14.000 rpm for 90 minutes in an eppendorf centrifuge. The HSV pellets were resuspended in coating buffer at a concentration of 1x10^8 pfu/ml. The viruses and different recombinant HIV-1 gp120 glycoproteins (5 µg/ml in coating buffer) were coated onto Maxisorp plate (NUNC, Denmark) overnight at 4°C. After blocking the plate with 5% BSA in TSM (20 mM Tris, 150 mM NaCl, 1 mM CaCl_2, 2 mM MgCl_2 pH 8.0) for 30 minutes at 37 °C, Jurkat-langerin lysate (1x10^8 cells/ml) was added (1:2 lysate:TSA) and incubated for 2 hours at room temperature. After washing, the plate was incubated with the specific non-blocking anti-Langerin antibody DCM4 (10µg/ml) for 1 hour. Subsequently, the plate was incubated with a goat anti-mouse peroxidase conjugate and binding was detected using ELISA. To determine specificity for the binding site of Langerin, the lysate was pre-incubated with mannan (1mg/ml) or EGTA (10mM) for 1 hr at 37°C.

**Fluorescent HIV-1 gp120 bead adhesion competition assay.** HIV-1 gp120 beads were produced as described previously. The adhesion assay was performed as follows: 5x10^4 Raji or Raji-Langerin cells were seeded in a v-bottom 96 wells plate (Greiner Bio-One) in TSA (0,5% BSA in TSM). The cells were pre-incubated with mannan (1 mg/ml), EGTA (10 mM) or HSV-2 (1x10^7 PFU/ml) for 15 min at 37°C. Next, the cells were incubated with beads for 45 min at 37°C. Binding was measured by flow cytometry.

**HIV-1 transmission.** moDCs and emigrant LCs were infected with HSV-1 syn 17+ or HSV-2 (MOI 1) overnight. The cells were plated at a concentration of 5x10^4 cells/well in V-bottom 96-well plates (Greiner Bio-One) and non-infected cells were pre-incubated or not with mannan (1mg/ml) for 30 minutes at 37°C. Next, the cells were incubated with JC-CSF (50 TCID) in medium containing acyclovir (50µM). After 2 hours, cells were washed twice with medium, and incubated with 2x10^5 activated CD4+ T cells in a flat bottom 96-well plate (Greiner Bio-One). Every 3 days new acyclovir was added to the wells to inhibit HSV infection. To determine transmission, supernatants were harvested at different days and frozen at -20°C until the concentration of HIV-1 capsid (p24) was measured by ELISA, as previously described.

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**References**

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