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TNF-α and TLR agonists increase susceptibility to HIV-1 transmission by human Langerhans cells ex vivo


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Genital coinfections increase an individual’s risk of becoming infected with HIV-1 by sexual contact. Several mechanisms have been proposed to explain this, such as the presence of ulceration and bleeding caused by the coinfecting pathogen. Here we demonstrate that Langerhans cells (LCs) are involved in the increased susceptibility to HIV-1 in the presence of genital coinfections. Although LCs are a target for HIV-1 infection in genital tissues, we found that immature LCs did not efficiently mediate HIV-1 transmission in an ex vivo human skin explant model. However, the inflammatory stimuli TNF-α and Pam3CysSerLys4 (Pam3CSK4), the ligand for the TLR1/TLR2 heterodimer, strongly increased HIV-1 transmission by LCs through distinct mechanisms. TNF-α enhanced transmission by increasing HIV-1 replication in LCs, whereas Pam3CSK4 acted by increasing LC capture of HIV-1 and subsequent trans-infection of T cells. Genital infections such as Candida albicans and Neisseria gonorrhoea not only triggered TLRs but also induced TNF-α production in vaginal and skin explants. Thus, during coinfection, LCs could be directly activated by pathogenic structures and indirectly activated by inflammatory factors, thereby increasing the risk of acquiring HIV-1. Our data demonstrate a decisive role for LCs in HIV-1 transmission during genital coinfections and suggest antiinflammatory therapies as potential strategies to prevent HIV-1 transmission.

Introduction

HIV-1 infection, the causative agent of AIDS, is still on the rise. It was estimated that 2 to 4 million people acquired HIV-1 in 2007, while already 31 to 36 million people are HIV-1 infected worldwide (1). Thus, the HIV-1 pandemic, for which there is currently no cure or vaccine available, is still growing. Heterosexual transmission of HIV-1 across genital epithelial tissue is the primary route of HIV-1 dissemination worldwide (1, 2). Therefore, increased knowledge about how sexual transmission of HIV-1 occurs and the identification of factors facilitating or enhancing this process are essential for the development of effective strategies to reduce new infections.

Many biological factors are involved in sexual transmission of HIV-1, and the risk of HIV-1 acquisition varies depending on these factors, including viral loads (3), viral variants, and host susceptibility, which may include contraception, male circumcision, and genital coinfection (2). Moreover, different soluble factors in body fluids might influence transmission such as factors in breast milk (4) and semen (5). Furthermore, genital coinfections have been linked to increased susceptibility to HIV-1 and include ulcerative sexually transmitted diseases (STDs) such as genital herpes, gonorrhea, syphilis, and chlamydial infections (6) as well as yeast and bacterial vaginal infections (7–9). However, the mechanisms accounting for increased HIV-1 susceptibility in the presence of genital coinfections are unclear. It is argued that these infections increase susceptibility by recruiting HIV-1 target cells into the site of infection (10) or by causing ulceration and subsequent bleeding (6). In addition, we hypothesize that pathogenic structures or inflammatory cytokines that are induced upon infection change the function of key players in the transmission of HIV-1 (11).

Langerhans cells (LCs) are a subset of DCs that reside in the epidermis of skin and in mucosal epithelia such as ectocervix, vagina, and foreskin (12, 13). LCs are therefore likely to be the first cells that encounter HIV-1 upon sexual transmission. However, there is debate about whether LCs are also the first cells infected by HIV-1 and whether they are involved in the initiation of systemic disease (12, 14–16). Several ex vivo skin explant studies have shown that LCs are susceptible to HIV-1 and transmit HIV-1 to T cells (12, 17, 18). We have recently demonstrated that HIV-1 infection of LCs and subsequent transmission to T cells is an inefficient process. Langerin, a C-type lectin specifically expressed by LCs, captures HIV-1 and acts as a protective barrier for HIV-1 infection by targeting HIV-1 to Birbeck granules for degradation. However, when the Langerin function is blocked or saturated using high virus concentrations, the virus is degraded or transmitted, and we hypothesize that its activation state and the encountered viral loads are a decisive factor.

Both inflammatory cytokines and pathogen-associated molecular patterns (PAMPs) induce LC activation (19–21), and these factors, which are present during coinfections, might breach the protective function of LCs to allow HIV-1 transmission. PAMPs are recognized by TLRs, and TLR triggering on LCs results in LC maturation (21). Here we set out to investigate the effect of bacterial and fungal coinfections on HIV-1 transmission by LCs. To mimic the

Nonstandard abbreviations used: LC, Langerhans cell; LTA, lipoteichoic acid; Pam3CSK4, Pam3CysSerLys4.

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Figure 1
An ex vivo skin model to investigate the effect of inflammation on HIV-1 transmission is shown. (A–C) Epidermal sheets were floated on medium in a 24-well plate and where indicated stimulated with different stimuli. After 6 hours the sheets were inoculated with HIV-1–eGFP. After 3 days, the epidermal sheets were removed and CCR5+ Jurkat T cells were added for an additional 7 days. Filled dendritic forms indicate LCs; open dendritic forms indicate GFP+–infected LCs; filled circles indicate CCR5+ Jurkat T cells; open circles indicate GFP+–infected T cells. (B) The migrated epidermal cells (day 3) were stained with antibodies against CD1a, Langerin, CD86, and CD3 and analyzed by flow cytometry to determine their phenotype and HIV-1 infection. Gates are based on isotype control (Supplemental Figure 1). R1 is gated on the larger cells in the population. Open histograms represent isotype-control; filled histogram specific antibody staining; the mean ± SD of the specific staining is depicted in the upper-right corner. The percentage of GFP+, CD3+, and CD1a+ cells ± SD is depicted in the upper-right corner of the dot plots. (C) Samples of the cocultures (day 5, 7, and 10) that were not stimulated with any stimulus were analyzed for GFP expression by flow cytometry. The percentage of GFP+ cells ± SD is depicted in the upper-right corner. As control for HIV-1 infection, the same concentration of HIV-1–eGFP was added to wells without epidermal sheets and processed similarly as the other conditions.
Results

An ex vivo model to determine the effect of coinfection on HIV-1 transmission. Bacterial and fungal coinfections of the genital tract result in increased risk of HIV-1 infection (6, 9, 22). To investigate the effect of different bacterial pathogens on HIV-1 transmission, we set up an ex vivo HIV-1 transmission model using human epidermal sheets (Figure 1A). The advantage of this model is that LCs reside in their physiological cellular environment, which is essential for LC function (23). Upon culture of the epidermal sheets, LCs migrated out of the epidermis, similar to LCs in the epithelial tissues that migrate toward the T cell–rich areas upon activation (12).

We have used the recombinant R5-tropic HIV-1 strain NL4.3-eGFP-BaL, expressing the eGFP upon replication (referred to as HIV-1–eGFP) (24), to investigate transmission of HIV-1 by LCs ex vivo by adding the virus to epithelial sheets. LC infection by HIV-1 is relatively inefficient and high virus concentrations are needed to induce infection (16, 25). At later time points, infected cells were detected by measuring GFP expression using flow cytometry, and viral infection of T cells was low but detectable after 7 days (mean of 1.8%; Figure 1C). At high virus concentrations, a small amount of residual virus remained infectious after 3 days and could infect CCR5

Figure 2
Different pathogens and pathogenic ligands induce TNF-α production in skin and vaginal biopsies. Skin or vagina epithelial biopsies were treated with Candida albicans, Neisseria gonorrhoea (A and B), Pam3CSK4, LTA, LPS, or flagellin (C). After 24 hours, the supernatant was collected, and TNF-α production was measured by ELISA. Error bars represent the mean ± SD of duplicates. For each panel, the different donors were measured in 2 independent experiments.
reproductive tract infections (28, 29). After 24 hours supernatant was harvested and TNF-α production was analyzed. The cytokines were measured after 24 hours when only a small number of cells had migrated out of the tissue, strongly indicating that the main effects of the pathogens are due to interactions with residing cells. Both Candida and Neisseria induced TNF-α production in epidermal and vaginal biopsies (Figure 2, A and B). Notably, TLR agonists LPS, lipoteichoic acid (LTA), Pam3CSK4, and flagellin also induced TNF-α production in the skin biopsies, indicating that TLR triggering mediates the production of TNF-α (Figure 2C).

In addition to TNF-α, we analyzed the production of the inflammatory cytokines IL-6 and IL-1β and the chemokine IL-8. IL-6 and IL-8 were both highly produced in skin biopsies. However, no differences were observed between unstimulated and stimulated tissues after 24 hours (Supplemental Figure 2, D and E) or 48 hours of culture (L. de Witte and M.A.W.P. de Jong, unpublished observations). Furthermore, the production of IL-1β in all skin biopsies was below the detection limit (<5 pg/ml; L. de Witte and M.A.W.P. de Jong, unpublished observations).

Candida albicans can switch between the yeast and hyphal form, which can differently affect immune responses and, moreover, differences between immune activation by live and heat-killed Candida have been observed (30). Therefore, we have compared the cytokine responses induced by heat-killed yeast with those of the live hyphal and yeast forms of Candida albicans. The live yeast form was obtained by treating Candida albicans yeasts with Amphotericin B, which prevents growth and thereby differentiation into the hyphal form. All Candida albicans forms resulted in TNF-α production, while IL-8 remained unchanged (Supplemental Figure 2A). Live Candida albicans treated with Amphotericin B enhanced IL-6 production significantly compared with the medium control, but this increase was not significant compared with the other stages of Candida albicans (Supplemental Figure 2B).

Thus, bacterial and fungal pathogens associated with genital tract infections induce TNF-α production both in epithelial and vaginal tissues and this can affect HIV-1 transmission.

TNF-α and TLR ligands affect HIV-1 transmission ex vivo. Next, we investigated whether TNF-α or TLR agonists influence HIV-1 transmission by LCs in the ex vivo transmission model. Extracellular bacterial and yeast microbes are mainly recognized by TLR2 (in combination with TLR1 or TLR6), TLR4, and TLR5 (28, 31). Therefore, we used the TLR agonists Pam3CSK4 (TLR1/TLR2), LTA (TLR2/TLR6), LPS (TLR4), and flagellin (TLR5). Epidermal sheets were preincubated with TLR agonists or TNF-α and subsequently infected with HIV-1–eGFP. At day 3 CCR5+ Jurkat T cells were added and infection was followed in time by fluores-
cent microscopy and flow cytometry. Almost no infected cells were detected in the medium control condition, confirming that HIV-1 transmission to T cells is not efficient under normal conditions, even though we used high concentrations of virus (Supplemental Figure 3 and Figure 3A). Strikingly, many syncytia were observed in the TNF-α-treated cultures at day 7, and high levels of GFP expression were detected in the T cells by fluorescent microscopy and flow cytometry (Figure 3A and Supplemental Figure 3). Further analyses by flow cytometry demonstrated that in the TNF-α-treated condition up to 50% of the T cells were infected (Figure 3, A and B). Although the variation between donors ranged from 25% to 50%, the increase in HIV-1 transmission by TNF-α was observed for all donors (Figure 3B).

The TLR1/TLR2 ligand Pam3CSK4 also increased HIV-1 transmission to T cells (Supplemental Figure 3 and Figure 3A) and about 5%–50% of the T cells were infected (Figure 3, A and B). Although the increased transmission was observed for all donors and did increase over time, the effect was more variable compared with the effect of TNF-α (Figure 3B). TLR agonists LTA, LPS, and flagellin marginally increased HIV-1 transmission in some donors (Figure 3, A and B). Enhancement of HIV-1 transmission by the different stimuli was dependent on interaction of HIV-1 with the epidermis, since transmission was not increased by the different stimuli in absence of epidermal sheets (Figure 3A). The increased transmission in the presence of TNF-α and Pam3CSK4 was statistically significant ($P < 0.001$ and $P < 0.01$, respectively), while overall, the effect of other TLR agonists was not significant (Figure 3B). To validate that GFP expression upon infection with HIV-1–eGFP correlates with the amount of virus produced, viral particles in the supernatant were quantified by ELISA (Figure 3C). The ex vivo experiment was performed as described before; however, migrated cells were washed before addition of the CCR5 Jurkat T cells to remove dead virus particles of the initial inoculum. Only low amounts of HIV-1 p24 were detected in the untreated sheets, whereas, as expected based on the GFP expression, high amounts of viruses were detected after TNF-α and Pam3CSK4 treatment. These data demonstrate that GFP expression correlates with viral production (Figure 3C). Transmission to T cells by LCs from 4 different donors was followed over time (Figure 3D). The TLR5 agonist, flagellin, increased HIV-1 transmission in 3 donors, whereas LPS enhanced HIV-1 transmission in 1 out of 4 donors (Figure 3D). LTA did not enhance HIV-1 transmission. Thus, the effects of TLR agonists flagellin and LPS are donor dependent.

**Figure 4**

TNF-α and Pam3CSK4 enhance HIV-1 transmission ex vivo. (A) Epidermal single-cell suspensions were stimulated with TNF-α and Pam3CSK4, and after 2 hours, the cells were inoculated with HIV-1–eGFP. After 2 hours the cells were washed and CCR5 Jurkat T cells were added for 7 days and analyzed for GFP expression by flow cytometry. A representative experiment out of 2 donors is depicted. (B) Epidermal sheets were stimulated with TNF-α, Pam3CSK4, LTA, LPS, or flagellin for 6 hours, and ex vivo transmission was determined as in Figures 1 and 3. A part of the migrated cells was extensively washed before addition of the CCR5 Jurkat T cells (right panel). HIV-1 transmission is depicted as percentage of CCR5 Jurkat T cells positive for GFP expression. Error bars represent the mean ± SD of duplicates. A representative experiment out of 2 donors is shown.

To further investigate a specific role for LCs during HIV-1 transmission ex vivo and to exclude direct effect of the TLR ligands,
produced soluble factors, or cell-free HIV-1 on T cells, the migrated cells were washed before addition of the CCR5
+ Jurkat T cells. Pam3CSK4 and TNF-α increased HIV-1 transmission with the same fold increase in this setting (Figure 4B), indicating that these compounds affect LC function in HIV-1 transmission. However, the percentage of infection was lower compared with the conditions that were not washed, indicating the presence of de novo synthesized virus in the medium or the presence of soluble factors that act on the target cells. The effect of flagellin was abrogated after washing the migrated cells (Figure 4B), indicating that flagellin directly or indirectly acts on the CCR5
+ Jurkat T cells rather than on the LCs. To further confirm that Pam3CSK4 and TNF-α increase HIV-1 transmission by LCs and to exclude effects of keratinocytes or minor T cell contaminations (4.3% ± 4.5% T cells) (Figure 1), we isolated the CD1a
+ LC fraction mediated HIV-1 transmission (Figure 4C), suggesting that contaminating T cells or keratinocytes are not involved in the observed effect of TNF-α and Pam3CSK4 on transmission. CD1a
+ isolation resulted in a reduction of 50% ± 8.5% of the T cells (data not shown), and we observed a low percentage of LC–T cell clusters (0.47% ± 0.42%). To further investigate the contribution of the LCs, we depleted the CD3
+ population, including the LC–T cell clusters. After depletion of the T cells and LC–T cell clusters, there was still a strong increase in transmission in the presence of TNF-α or Pam3CSK4 (data not shown). Thus, using the ex vivo transmission model we demonstrate that TNF-α and Pam3CSK4 increase HIV-1 transmission by LCs to T cells.

TNF-α enhances HIV-1 replication in LCs. Next we investigated whether the TNF-α- and Pam3CSK4-mediated increase in HIV-1 transmission is the consequence of an increase in LC infection, since HIV-1 transmission by LCs under steady-state conditions depends on direct infection of LCs with HIV-1 (16, 34). Epidermal sheets were inoculated with replication-competent HIV-1–eGFP in the presence of TNF-α or Pam3CSK4, and the infection of LCs was determined by flow cytometry at day 3 (Figure 5, A and B). Low infection of LCs was detected without stimuli (Figure 5, A and B) as demonstrated previously (16). Although donor variations were observed, both TNF-α and Pam3CSK4 increased LC infection compared with the medium control (Figure 5B). Thus, both stimuli increase LC infection, which contributes to the observed increase in HIV-1 transmission by LCs (Figure 3). We have investigated the expression of CD4, CCR5, and Langerin after ex vivo stimulation of the cells with Pam3CSK4 and TNF-α. The increased infection was independent of expression of the entry receptors CD4 and CCR5 or the expression of the protective receptor Langerin, since the expression levels did not significantly change after ex vivo stimulation (Supplemental Figure 4). Langerin could also affect HIV-1 transmission after LC infection. However, no differences in Langerin expression were observed between noninfected and infected cells.
gerin expression were observed after HIV-1 infection in presence of Pam3CSK4 and TNF-α (M.A.W.P. de Jong and L. de Witte, unpublished observations). Notably, the fold increase in percentage of infected LCs after stimulation with either TNF-α or Pam3CSK4 was less pronounced than the fold increase in transmission, indicating that LCs produce more virus progeny due to increased replication or that LCs transmit initial captured virus particles. First, we investigated whether the stimuli induced HIV-1 replication in LCs by measuring the presence of the multiply spliced mRNAs encoding the HIV-1 early genes tat and rev with quantitative real-time PCR, which is a method to determine HIV-1 replication (35). Next, we investigated whether viral capture was altered by the stimuli, by measuring the amount of full-length viral RNA after 6 hours as a measure for viral particle copies. Epidermal single-cell suspensions contained 1.16% ± 1.74% CD3+ T cells (n = 8) and were incubated with HIV-1 in presence or absence of TNF-α and Pam3CSK4. After 6 hours, the cells were washed extensively and lysed for mRNA capture. Both TNF-α and Pam3CSK4 increased the production of Tat/Rev transcripts (Figure 5C), demonstrating that both stimuli induce HIV-1 production in LCs. LCs treated with Pam3CSK4 contained a high amount of full-length viral RNA (Figure 5D), strongly suggesting that Pam3CSK4 increases HIV-1 capture by LCs. We did not observe any increase in full-length viral RNA in TNF-α–treated LCs, confirming that TNF-α solely enhanced HIV-1 replication and not HIV-1 capture. Next, we determined the ratio between Tat/Rev transcripts and full-length viral RNA in the different LCs as a measure for active HIV-1 replication per viral copy. Notably, TNF-α induced a strong upregulation of HIV-1 replication per viral copy, whereas Pam3CSK4 did not increase or in some donors decreased HIV-1 replication per viral copy (Figure 5E). Similar results were obtained with emigrated LCs (M.A.W.P. de Jong and L. de Witte, unpublished observations), demonstrating that the isolation procedure does not interfere with the observed results. These data suggest that the increased Tat/Rev transcription observed in Pam3CSK4-treated LCs is due to increased HIV-1 capture and integration but not to increased rate of replication, in contrast to TNF-α treatment that solely enhances HIV-1 replication (Figure 5). Thus, TNF-α and Pam3CSK4 induce HIV-1 infection of LCs through increased replication and viral uptake, respectively.

**Figure 6**
Pam3CSK4 enhances trans-infection of HIV-1. (A and B) Epidermal sheets or (C–E) epidermal single-cells suspensions were stimulated with TNF-α or Pam3CSK4 (E) after replication-competent HIV-1–eGFP to show where the virus is used. After 6 hours the sheets/cells were inoculated with replication-competent HIV-1–eGFP or single-cycle HIV-1–eGFP (HIV-1 NL4.3-eGFPΔ envelope pseudotyped with the BaL envelope). After 3 days, the epidermal sheets were removed or the cell suspensions were washed and CCR5+ Jurkat T cells were added. At day 5, the cocultures were analyzed for GFP expression by flow cytometry. (A) The response to different virus doses is depicted. (B and D) The mean of the responses of different donors are depicted, the values were analyzed by ANOVA for statistical differences. **P < 0.01. Error bars represent the mean ± SD of duplicates.
LCs do not efficiently mediate trans-infection (16), and transmission is mainly dependent on de novo virus production.

We investigated whether infection of activated LCs is indeed essential to the observed increased HIV-1 transmission using replication-defective HIV-1 (BaL-pseudotyped NL4.3-eGFP Δ envelope) in the ex vivo transmission model. Epidermal sheets were inoculated with replication-defective HIV-1 in the presence of TNF-α or Pam3CSK4, and transmission to CCR5+ Jurkat T cells was determined. Notably, Pam3CSK4 increased HIV-1 trans-infection to T cells (Figure 6, A and B). TNF-α slightly enhanced trans-infection in some donors; however, this was not significant between the different donors (Figure 6B), demonstrating that Pam3CSK4 and TNF-α increase HIV-1 transmission through different mechanisms. We measured trans-infection of single-cycle HIV-1 by LCs from an LC-enriched skin epidermal single-cell suspension. Similar results were obtained using these cell suspensions (Figure 6, C and D), demonstrating that HIV-1 trans-infection was not dependent on increased LC migration.

Next, we compared transmission of single-cycle HIV-1 with replication-competent HIV-1. Pam3CSK4 increased transmission of both viruses to similar levels, suggesting that a major part of the increase of HIV-1 transmission by Pam3CSK4-stimulated LCs is mediated by trans-infection (Figure 6E). Thus, Pam3CSK4 but not TNF-α enhances trans-infection of HIV-1 by LCs.

Pam3CSK4 increases HIV-1 capture by LCs. To further investigate the mechanisms that are involved in the increased trans-infection after Pam3CSK4 stimulation, we investigated the interaction of HIV-1 and Pam3CSK4-stimulated LCs using immunofluorescence experiments.
microscopy. Emigrant LCs were stimulated for 1 hour, and subsequently, inoculated with HIV-1 for 2 hours. Pam3CSK4 induced clustering of the emigrant LCs to clusters of 5–30 cells (Supplemental Figure 5A), and the cells had a more polarized morphology (Supplemental Figure 5B), indicating that the cells are activated. In unstimulated cells, single HIV-1 particles were observed in approximately 1 of 10 cells (different positive cells depicted in 7A and Supplemental Figure 6). Strikingly, multiple HIV-1 particles were observed in about 30% of the Pam3CSK4-stimulated cells (Figure 7, A and B, and Supplemental Figure 6). HIV-1 was often observed in clusters both on the cell membrane as well as in intracellular vesicles (Figure 7B and Supplemental Figure 6B). These data further confirm that Pam3CSK4 increases capture of HIV-1 by LCs. Exosomes of DCs have been implicated in transmission of HIV-1 (37). We therefore analyzed whether HIV-1 in Pam3CSK4-stimulated cells colocalized with HLA class I, molecules that are abundantly present in exosomes (37). In some cells, colocalization of HIV-1 and HLA class I was observed; however, virus was also observed in clusters on the cell membrane that did not colocalize with HLA class I (Supplemental Figure 7). Thus, in Pam3CSK4-stimulated cells HIV-1 is present both intracellularly and on the cell membrane.

To investigate whether intracellular or extracellular HIV-1 is transmitted to the target cells, we treated the epidermal single-cell suspension with trypsin after incubation with the single-cycle HIV-1. Strikingly, trypsin treatment abrogated the increased transmission induced by Pam3CSK4 (Figure 7C), demonstrating that primarily cell-bound HIV-1 is transmitted after Pam3CSK4 treatment. To confirm these results, we selectively neutralized cell-surface bound HIV-1 using the broadly anti-HIV-1 neutralizing antibody b12 that binds to HIV-1 gp120 and prevents binding to CD4 or chemokine receptors (38). Pam3CSK4-treated LCs were incubated with HIV-1 for 2 hours at 37°C, washed, and subsequently treated with the neutralizing antibody b12 at 4°C to neutralize cell-bound but not internalized HIV-1. Strikingly, addition of this antibody completely abolished the transmission induced by Pam3CSK4 (Figure 7D), without affecting the viability (data not shown). Therefore, these data demonstrate that Pam3CSK4 increases HIV-1 binding to LCs and transmission of these mostly cell-bound virus particles to target cells. The high viral concentrations needed in the ex vivo model to investigate transmission have been shown to saturate the protective function of Langerin (16). Therefore, we investigated whether TNF-α and Pam3CSK4 enhance transmission at low viral titers using isolated LCs. At low viral concentrations, Pam3CSK4, in contrast to TNF-α, strongly induced HIV-1 transmission to T cells (Figure 7E), suggesting that Pam3CSK4 overcomes the protective Langerin function. In contrast, TNF-α was only able to enhance HIV-1 transmission at those virus concentrations that already allowed transmission, supporting our data that TNF-α-mediated transmission is dependent on primary LC infection.

**Discussion**

In order to reduce the growing HIV-1 pandemic, there is a strong pressure to understand HIV-1 transmission. Sexual transmission of HIV-1 is the major route of infection worldwide (1), and little is known about the factors governing HIV-1 transmission and susceptibility to HIV-1. Genital coinfection is a risk factor for acquiring HIV-1 (2), indicating that pathogens or host responses to the pathogens create a genital environment that favors HIV-1 transmission. Here we demonstrate a potential role for LCs in the increased susceptibility to HIV-1 during fungal and bacterial coinfections.

Due to their epithelial localization and function, LCs are the first cells to encounter HIV-1 in the genital epithelial tissues (12, 13). Several studies suggest that HIV-1 subverts the function of LCs for viral transmission to T cells, thereby infecting the host (18, 34). However, other reports argue that not LCs but T cells and subepithelial antigen-presenting cells are the first cells infected by HIV-1 (14, 15, 39). Moreover, our recent data demonstrate that LCs are not efficiently infected by HIV-1 and form a barrier to HIV-1 infection through the function of the C-type lectin Langerin (16); Langerin captures HIV-1, resulting in efficient virus degradation, which prevents infection. However, Langerin inhibitors or high virus concentrations (>100 ng p24/10^5 LCs) that saturate Langerin allow LC infection and subsequent HIV-1 transmission (16). These data suggest that under normal conditions LCs function as a barrier but that specific conditions might change LC function and promote transmission. Using an ex vivo transmission model, we demonstrate here that indeed HIV-1 transmission by LCs is low and inefficient; high concentrations of HIV-1 (30 ng p24 per epidermal sheets, containing approximately 10^5 LCs) were needed to observe transmission by LCs. Strikingly, activation of LCs by TNF-α or Pam3CSK4 induces a strong increase in transmission of HIV-1, suggesting that under conditions of high viral loads and inflammation, LCs play a pivotal role in HIV-1 transmission.

Inflammatory conditions induce LC maturation, which includes downregulation of antigen capture and processing as well as increased migration from the periphery to the lymphoid tissues. Monocyte-derived LC-like cells are more susceptible to HIV-1 infection after activation with CD40L (40). Moreover, a recent study demonstrated that CD34^+ derived LC-like cells only efficiently transfer HIV-1 after activation by a combination of LPS and TNF-α (41). Although primary LCs are unresponsive to LPS (21), these studies using LC-like models suggest that LC activation is an important determinant in whether LCs protect against HIV-1 infection or transmit the virus to T cells. Moreover, the cellular environment might also be pivotal to LC function in HIV-1 transmission. Therefore, we have used an ex vivo skin model to investigate the role of primary LCs and the environment in HIV-1 transmission during coinfections and inflammatory conditions.

Although the LC fractions contained a small percentage of T cells, we were able to demonstrate the specific contribution of LC by using CD1a isolation and CD3 depletion.

Coinfections with pathogens activate LCs through TLR triggering. Therefore, we investigated whether different bacterial TLR agonists influence HIV-1 transmission in the ex vivo model. We demonstrated that TLR2, TLR4, and TLR5 agonists increase HIV-1 transmission in tissues derived from some donors, whereas no increase was observed in other donors. Notably, the TLR4 agonist LPS did induce HIV-1 transmission by LCs in some donors, even though LCs do not express TLR4 (L. de Witte and M.A.W.P. de Jong, unpublished observations) (20, 21). These data suggest that the observed increase for at least the TLR4 agonist is due to an indirect activation of LCs, such as production of proinflammatory cytokines by keratinocytes, since keratinocytes and epithelial cells do express TLR4 (20). Indeed, we observed the production of TNF-α after LPS stimulation of skin biopsies. This might be variable throughout donors due to different levels of TLR expression and activity, resulting in the variation in HIV-1 susceptibility (16).

The TLR5 agonist flagellin enhanced HIV-1 transmission in our
ex vivo model in half of the donors. However, the effect of flagel-
lin was indirect, since the increased transmission was abrogated
after washing the emigrated LCs and flagellin did not increase
HIV-1 capture or replication in LCs as determined by RT-PCR
(L. de Witte and M.A.W.P. de Jong, unpublished observations).
Moreover, flagellin itself did not increase susceptibility of CCR5-
Jurkat T cells (L. de Witte and M.A.W.P. de Jong, unpublished
observations), suggesting that flagellin induces the production of
a soluble factor by surrounding cells that enhances infection of
T cells. Furthermore, TNF-α neutralization by antibodies in the
ex vivo experiment did not prevent the increase of transmission by
flagellin (Supplemental Figure 8), suggesting that TNF-α is not
involved in the enhanced transmission by flagellin.

In contrast to the other TLR agonists, Pam3CSK4 enhanced
transmission in all donors tested. Pam3CSK4 is a synthetic tripal-
mitoylated lipopeptide that mimics the acetylated amino termi-
nus of bacterial lipoproteins (42, 43). Recognition of Pam3CSK4
is mediated by a dimer of TLR1 and TLR2 (44). LCs and kerato-
cytes both express TLR1 and TLR2 (20, 21, 45). Neutralizing anti-
bodies against TNF-α did not abrogate the Pam3CSK4 induction
of HIV-1 transmission (Supplemental Figure 8), demonstrating
that Pam3CSK4 increased transmission independent of TNF-α.
Using a single-cycle replication-defective HIV-1, we demonstrate
that the increase in HIV-1 transmission by Pam3CSK4-stimulated
LCs is independent of HIV-1 infection of LCs, demonstrating that
the increase is at least partly due to trans-infection of T cells; HIV-1
capture by LCs is increased and the surface-bound viruses are effi-
ciently transmitted to T cells. This is supported by our finding
that HIV-1 capture by LCs is strongly increased after Pam3CSK4
stimulation. Single particles and clusters of HIV-1 are observed
intracellularly and on the cell membrane, some colocalizing with
HLA class I molecules, which might reflect exosomes. Although
HIV-1 was observed in intracellular vesicles, cell-bound HIV-1 was
primarily transmitted after Pam3CSK4 stimulation. Pam3CSK4
might upregulate the expression of receptors that increase HIV-1
capture, such as heparan sulfate proteoglycans (46). However,
heparinase treatment did not abrogate HIV-1 transmission after
Pam3CSK4 treatment (L. de Witte and M.A.W.P. de Jong, unpub-
lished observations), indicating that heparan sulfates are not
involved. Possibly, Pam3CSK4 enhances LC activation, which
results in increased HIV-1 binding. Notably, enhanced HIV-1 cap-
cture by Pam3CSK4 is observed within 30 minutes (L. de Witte and
M.A.W.P. de Jong, unpublished observations) and Pam3CSK4-
stimulated LCs rapidly change phenotypically and form clusters.
A recent report demonstrated that Pam3CSK4 stimulation in T cells
results in the activation of NF-κB and upregulation of the expres-
sion of CCR5. After 24 hours of stimulation, these activated T cells
are more susceptible to both X4- and R5-tropic HIV-1 (47). How-
ever, we observed increased trans-infection, which is independent
of fusion of HIV-1 with LCs, strongly suggesting that increased
HIV-1 capture but not infection is responsible for transmission after
Pam3CSK4 stimulation.

TLR agonists as well as whole pathogens can increase HIV-1
transmission indirectly by changing the cytokine environment.
Indeed, TLR agonists, Neisseria gonorrhoea and the different forms
of Candida albicans induced the production of proinflammatory
cytokine TNF-α in vaginal and skin tissues ex vivo. Therefore, we
investigated what the effect of proinflammatory cytokine TNF-α
was on HIV-1 transmission. Our data demonstrate that TNF-α
strongly upregulated HIV-1 transmission by LCs ex vivo, suggest-
ing that LC activation by TNF-α leads to increased susceptibil-
ity to HIV-1 under coinfection circumstances. Further study of
the molecular mechanism demonstrates that TNF-α increased
the level of HIV-1 replication in infected LCs, which results in
enhanced HIV-1 transmission of de novo produced viruses to
T cells. This is further supported by the need for HIV-1 replication
in LCs, since TNF-α – in contrast to Pam3CSK4-treated LCs do
not transmit replication-defective HIV-1 to T cells ex vivo. More-
over, TNF-α did not increase HIV-1 capture by LCs but enhanced
initiation of HIV-1 transcription as determined by the increase in
the multiply spliced transcripts of the early HIV-1 genes tat and
rev. These data are supported by previous studies demonstrating
that TNF-α enhances replication of HIV-1 in T cells and macro-
phages (27). These data strongly suggest that coinfections abro-
gate the protective barrier function of LCs and allow LC infec-
tion and subsequent HIV-1 transmission. Inflammation not only
affects LC function but also epithelial and sometimes subepi-
thelial tissues, resulting in the production of inflammatory fac-
tors, influx of immune cells, ulceration, and bleeding. Different
mechanisms have been proposed to explain the increased risk of
HIV-1 acquisition during coinfection such as genital ulceration
(6). However, we here demonstrate that the role of LCs should be
taken into account.

We have previously shown that Langerin protects LCs from HIV-1
infection and thereby prevents HIV-1 transmission (16). Here we
have used high viral concentrations in the ex vivo transmission
model, which were previously shown to saturate the Langerin
function (16). Titration of HIV-1 in the transmission assays sug-
gen that Pam3CSK4 but not TNF-α activation overcomes the
protective function of Langerin. Pam3CSK4 induced viral trans-
mission by LCs even at low concentrations when Langerin was not
saturated. In contrast, TNF-α only increased HIV-1 transmission
at concentrations that already allowed HIV-1 transmission under
normal conditions. Since TNF-α enhances HIV-1 transcription,
low levels of infection are a prerequisite for TNF-α to enhance
HIV-1 transmission and therefore dependent on the Langerin
function. In contrast, Pam3CSK4 enhances capture of HIV-1 for
transmission to T cells. Therefore, at low viral loads, there could
be competition between (unknown) surface attachment receptors
and Langerin for HIV-1. Our data suggest that LC activation by
Pam3CSK4 is strong enough to negate the protection by Langerin.
From the observed mechanisms, we speculate that the presence
of TNF-α is extremely important when Langerin function has been
compromised and initial infection has taken place. In contrast,
TLR1/TLR2 activation by pathogens during coinfections will
overcome the Langerin barrier and induce HIV-1 transmission by
LCs through increased HIV-1 uptake and transmission to T cells,
thereby strongly enhancing the susceptibility of the host to HIV-1
infection. In our model, no differences in surface Langerin expres-
sion were observed after addition of TNF-α or Pam3CSK4. How-
ever, we cannot exclude that the degradation function or cluster-
ing of Langerin is hampered in the presence of these compounds.
Further studies will be necessary to investigate the effects of LC
activation on Langerin function.

Since the development of an effective vaccine or a curative treat-
ment for HIV-1 is not progressing rapidly, the WHO stated that
focus should be on the development of an agent that prevents
HIV-1 transmission and that can be applied topically, such as a
genital cream (1). Our results indicate that coinfection results in a
changed LC phenotype that can result in transmission, mediated
through different mechanisms. To prevent LC infection and subsequent transmission, it is attractive to try to block the entry receptors of HIV-1 on the LCs, such as CD4 and CCR5 (48). However, TLR activation by coinfected pathogens might increase HIV-1 transmission by yet unknown mechanisms, such as the increased capture of HIV-1 and subsequent trans-infection observed with Pam3CSK4 treatment. Since these mechanisms are potential targets for therapies these need to be further unravelled. Since TNF-α enhances LC infection and subsequent transmission, the cytokine itself or its receptor represents targets to prevent HIV-1 transmission. Moreover, these results emphasize the argument that prevention and fast intervention to treat coinfections and especially chronic coinfections should be taken as a serious point in the prevention of HIV-1 transmission.

**Methods**

**Antibodies and reagents.** The following reagents were used: tripalmitylated lipopeptide Pam3CSK4 (InvivoGen), LTα from *Staphylococcus aureus* (Sigma-Aldrich), LPS from *Salmonella Typhosa* (Sigma-Aldrich), flagellin derived from *Bacillus Subtilis* (InvivoGen), recombinant human TNF-α (Stratham Biotech), DCGM4-PE (anti-Langerin; Beckman Coulter Inc.), HI49-FITC (anti-CD1a; Pharmingen), goat anti-mouse-FITC (Zymed Laboratories Inc.), HA5.287-PE (CD86; Immunotech), 2D7-PE (anti-CCR5; Pharmingen), A-07751-PE (anti-CD4), anti–TNF-α (Biossion), KC57-RD1-PE (anti-HIV-1 p24; Beckman Coulter Inc.), HLA-ABC-m3-FITC (anti-HLA-B27; Abcam), NA1/34 (anti-CD1a; Dako Cytomation), IgG1 PE isotype (BD Biosciences), IgG1 isotype control (mouse IgG1; Biolegend), and HIV-1 neutralizing antibody b12 or an isotype control was added to the cells (20 μg/ml) for 30 minutes and subsequently incubated with 50 μl of a trypsin solution (0.05% trypsin in PBS) for 10 minutes. By washing 3 times in complete Iscove’s modified Dulbecco’s medium trypsin was inactivated. To neutralize cell-bound but not internalized HIV-1, neutralizing antibody b12 or an isotype control was added to the cells (20 μg/ml) for 30 minutes at 4°C to prevent internalization of the antibody. Cells were washed to remove unbound antibody and CCR5+ Jurkat T cells (2 × 10⁶ cells) were added. Infection was measured by flow cytometry.

**Trypsin treatment.** LCs (5 × 10⁶ cells) were preincubated with Pam3CSK4 (5 μg/ml) for 30 minutes and subsequently inoculated with Bal-pseudotyped NL4.3-eGFP envelope (50 ng p24 [2.5 × 10⁵ IU]/sheet) for 2 hours. To cleave off HIV-1, LCs were washed in PBS and subsequently incubated with 50 μl of a trypsin solution (0.05% trypsin in PBS) for 10 minutes. By washing 3 times in complete Iscove’s modified Dulbecco’s medium trypsin was inactivated. To neutralize cell-bound but not internalized HIV-1, neutralizing antibody b12 or an isotype control was added to the cells (20 μg/ml) for 30 minutes at 4°C to prevent internalization of the antibody. Cells were washed to remove unbound antibody and CCR5+ Jurkat T cells (2 × 10⁶ cells) were added, and transmission was determined by flow cytometry. Viability of the cells after trypsin treatment was determined by trypan blue staining and was more than 90%.

**Ex vivo stimulation.** Biopsies of fresh skin and vagina tissue were taken within 3 hours after the operation using a 6-mm biopsy punch (Microtek Medical). The biopsies were floated in a 48-well plate on 250 μl Iscove’s modified Dulbecco’s medium, 10% FCS, and 10 μg/ml gentamycine and stimulated with heat-killed or live *Candida albicans* (0.25 × 10⁶/ml) in the presence or absence of Amphotericin B (25 μg/ml) (53) and paraformaldehyde-inactivated *Neisseria gonorrhoea* LGTD (2.5 × 10⁵/ml), generously provided E.C. Gotschlich (Rockefeller University, New York City, New York, USA) (54), LPS (10 μg/ml), or LTA (10 μg/ml). After 24 hours, supernatant was collected and frozen. The samples were analyzed for different cytokines by ELISA (Biosource International).

**Viral replication.** Epidermal single cells (5 × 10⁶ cells) were preincubated with Pam3CSK4 (5 μg/ml) or TNF-α (0.1 μg/ml) for 30 minutes and inoculated with HIV-1 NL4.3-Bal (20 ng p24 [2 × 10⁵ IU]/sheet). After 6 hours, the cells were washed extensively with PBS, after which host mRNA and viral RNA were specifically 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 (55). Multiply spliced HIV-1 transcripts were detected with primers that detect both Tat and Rev mRNAs across an intron. Full-length viral RNA was detected with primers against the long-terminal repeat (LTR) US sequence (forward) and the gag coding sequence (reverse). The sequences are as follows: HIV-1 LTR-gag, forward, GTTGGAAAAATCTCTAGAGTTG, reverse, CGCTCCTC-GACCATCTC; HIV-1 Tat-Rev, forward, ATGCAGAAGAAAGCGGAG, reverse, ATTTTCCCAGTGGTAC. Specific primers for HIV-1 LTR, Tat/ Rev, and GAPDH (55) were designed by Primer Express 2.0 (Applied Biosystems). Transcription was adjusted for GAPDH transcription, and relative mRNA expression of HIV-1-infected control samples was set at 1.

**Immunofluorescence microscopy.** LCs (2 × 10⁶ cells) were preincubated with Pam3CSK4 (5 μg/ml) for 1 hour at 37°C. The cells were inoculated with HIV-1 NL4.3-Bal eGFP (30 ng p24 [12 × 10⁵ IU/sheet]) or the single-round Bal-pseudotyped NL4.3-eGFP envelope (10 or 100 ng p24/sheet; 5 × 10⁸ and 5 × 10⁷ IU/sheet). After 3 days, the cells were removed and CCR5+ Jurkat T cells (2 × 10⁶ cells) were added. Infection was monitored by flow cytometry, p24 ELISA, and immunofluorescence microscopy (original magnification, ×200; Leica DMIL fluorescence microscope; Leica Microsystems).

**LC transmission.** LC-enriched epidermal single-cell suspensions were generated as described (16). As determined by CD1a-staining, these single-cell suspensions generally contained 5%–10% LCs. Although trypsin digestion might result in loss of some CD4 expression (52), isolated LCs were infected with HIV-1 (16). Epidermal cells (1.5 × 10⁶ cells) were preincubated with Pam3CSK4 (5 μg/ml) or TNF-α (0.1 μg/ml) for 4 hours and inoculated with HIV-1 NL4.3-Bal-eGFP (20 ng p24 [8 × 10⁵ IU/sheet]) or the single-round Bal-pseudotyped NL4.3-eGFP envelope (50 ng p24 [2.5 × 10⁵ IU]/sheet). At day 3, cells were washed and CCR5+ Jurkat T cells (2 × 10⁶ cells) were added. Infection was measured by flow cytometry.

**Statistics.** Values for percentage of GFP-expressing CCR5+ Jurkat T cells for 1 donor are calculated as the mean of duplicate measurements and rep-
resented as the mean ± SD of the duplicate. To determine the variation in transmission among different donors, the mean values of GFP expression of CCR5+ Jurkat T cells of different donors were analyzed with ANOVA. When the overall F test was significant, differences among the donors were further investigated with the post-hoc Bonferroni test using Graphpad Prism software. A probability of P < 0.05 was considered statistically significant.

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