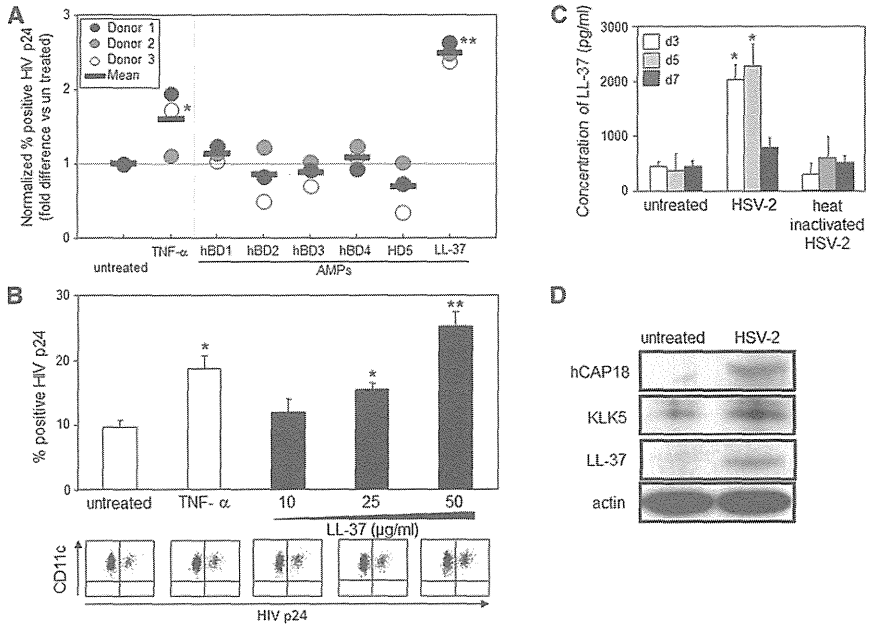


**Figure 3. LL-37 Enhances HIV Susceptibility in mLCs**

mLCs were stimulated with the indicated AMPs or rhTNF-α 24 hr prior to HIV exposure. To assess HIV infection levels, mLCs were collected 7 days after HIV exposure, and HIV $p24^+$ cells were quantified in $langerin^+$ $CD11c^+$ mLCs.

(A) Each circle indicates the normalized percentage of positive cells for HIV p24; mean values obtained from different donors are shown as horizontal marks.

(B) The percentage of positive cells for HIV p24 in $langerin^+$ $CD11c^+$ mLCs and representative flow cytometric analyses following LL-37 stimulation.

(C) NHEKs were exposed to HSV-2 or heat-inactivated HSV-2. Following culture for the indicated number of days, LL-37 levels were measured in supernatants by ELISA. Results are shown as means ± SD ($n = 3$) (*$p < 0.05$).

(D) NHEKs were treated with HSV-2 for 3 days and then lysed. The expression of hCAP18, KLK5, and LL-37 was determined by western blot analysis. All data shown represent at least two separate experiments. See also Figure S3.

of CD86, CD83, and CCR7 on mLCs (Figure S3), indicating that LL-37 induces LC maturation.

To confirm whether HSV-2-treated NHEKs could produce LL-37 protein, we measured LL-37 protein levels in culture supernatants from NHEKs treated with medium alone, HSV-2, or heat-inactivated HSV-2 by ELISA. HSV-2 significantly induced production of LL-37 in NHEKs, which peaked at day 5 (Figure 3C). Expression levels of kallikrein 5 (KLK5) have been shown to parallel induction of LL-37 in KCs, since the activity of cathelicidin is controlled by enzymatic processing of the proform hCAP18 to a mature peptide LL-37 by KLK5, a serine protease (Morizane et al., 2010; Yamasaki et al., 2006). Therefore, we measured protein levels of LL-37, hCAP18, and KLK5 in NHEKs treated with medium alone or HSV-2. As shown in Figure 3D, HSV-2 induced production of LL-37 in NHEKs. Similarly, HSV-2 increased expression of hCAP18 as well as KLK5, and these protein levels coincided with the induction of LL-37 in NHEKs (Figure 3D).

To further confirm the participation of LL-37 in this enhancement, we used RNA interference (siRNA) to block LL-37 production. Protein levels were quantified by western blotting followed by densitometry analysis. Transfection of siRNA targeting LL-37 induced an efficient knockdown in NHEKs (55% downregulation; Figure 4A). In line with the results of western blot analyses, siRNA-mediated interference of LL-37 in NHEKs significantly reduced enhancement of HIV infection in mLCs by supernatants from HSV-2-treated NHEKs, in comparison with control siRNA targeting an irrelevant sequence (Figure 4B). Based on these results, we conclude that enhanced HIV infection in mLCs by supernatants from HSV-2-treated NHEKs is, at least in part, mediated by LL-37.

Recently, TNF-α derived from KCs has also been shown to enhance HIV susceptibility of LCs (de Jong et al., 2008; Ogawa et al., 2009). In our experiments, however, TNF-α was not detected in culture supernatants from NHEKs treated by HSV-2 (data not shown), consistent with a recent report (de Jong et al., 2010). In addition, preincubation of supernatants from HSV-2-treated NHEKs with an anti-TNF-α neutralizing mAb, prior to exposing mLCs, did not affect HIV susceptibility in mLCs (Figure S4).

## LL-37 Enhances Surface Expression of CD4 and CCR5 on mLCs

Previous studies have revealed that langerin expressed on LCs is a natural barrier to HIV infection because HIV virions captured by langerin are internalized into LC Birbeck granules and degraded (de Witte et al., 2007). In addition, APOBEC3G (A3G) and SAM domain and HD domain 1 (SAMHD1) has been recently shown to function as a potent postentry cellular restriction factor for HIV in DCs or LCs (Hrecka et al., 2011; Laguette et al., 2011; Ogawa et al., 2009; Pion et al., 2006). Therefore, we next examined whether LL-37 affects the expression levels of these molecules in mLCs. LL-37 stimulation did not affect the expression of langerin, A3G, or SAMHD1 (Figures 4C and 4D and Figure S5). In contrast, LL-37 significantly increased surface expression of CD4 and CCR5 on mLCs (Figure 4C). In addition, siRNA-mediated interference of LL-37 in NHEKs significantly reduced the enhancement of surface expression of CD4 and CCR5 in mLCs by supernatants from HSV-2-treated NHEKs, in comparison with control siRNA targeting an irrelevant sequence (Figure 4C). Thus, our results suggest that LL-37 enhances HIV infection in LCs by increasing surface expression of HIV receptors, rather than by modulating restriction factors such as langerin, A3G, or SAMHD1.

Since LL-37 upregulated surface expression of CD4 and CCR5 in LCs, we next examined whether LL-37 specifically enhanced R5-tropic HIV entry into LCs by using single-round infection assays with pseudotyped viruses containing a luciferase reporter and different envelope proteins (Env): Env from either R5 HIV-1 (JR-FL; R5), X4 HIV-1 (IIIB; X4), or vesicular stomatitis virus (VSV-G). As expected, we found that LL-37 pretreatment enhanced the infectivity of mLCs to R5-VSV in

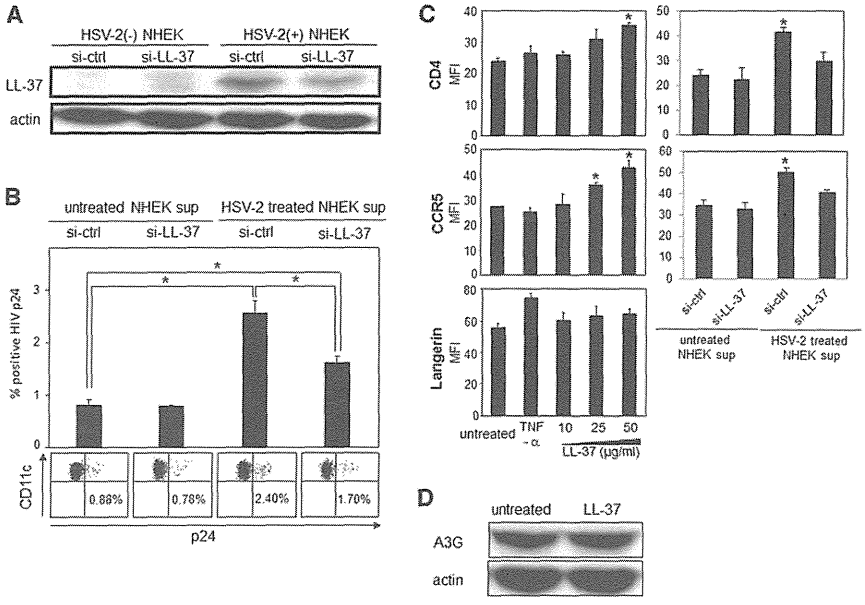


**Figure 4. Silencing of LL-37 in HSV-2-Infected NHEKs Abrogates Enhanced HIV Infectivity in mLCs**

(A) NHEKs were transfected with control or LL-37 siRNA and then exposed with or without HSV-2. Cells were lysed and then determined the expression of LL-37 by western blot analysis.

(B) mLCs were incubated with indicated culture supernatants for 12 hr and then exposed to R5 HIV. mLCs were collected 7 days after the HIV exposure, and HIV $p24^+$ cells were assessed in $langerin^+$ $CD11c^+$ mLCs. Representative flow cytometric analyses of CD11c and p24 mAb double-stained cells are shown.

(C) mLCs were stimulated with TNF-α or LL-37 at the indicated concentrations or indicated culture supernatants for 24 hr. The expression of CD4, CCR5, and langerin was assessed by flow cytometry.

(D) The expression of A3G was determined by western blot analysis. Results are shown as means ± SD (n = 3) (*$p < 0.05$). All data shown represent at least two separate experiments. See also Figure S4.

a dose-dependent manner, but LL-37 did not affect infection with VSV-G (Figure 5A). Consistent with previous findings (Kawamura et al., 2001), mLCs were resistant to X4-VSV, even after LL-37 treatment. These results provide direct evidence that LL-37, which upregulates surface expression of CD4 and CCR5 in LCs, promotes increased R5 HIV entry into these cells. Furthermore, similar effects of LL-37 were observed when mLCs were infected with R5 HIV primary isolates: JR-FL and AD8 (Figures 5B and 5C).

### LL-37 Decreases HIV Infectivity in mDCs

We next examined whether LL-37 affects HIV infectivity in non-LC-like DCs. Similar to mLCs, LL-37 significantly upregulated surface expression of CD86 and CCR7 on monocyte-derived DCs (mDCs, Figure S3), indicating that LL-37 induces DC maturation. In marked contrast to mLCs, however, there was inhibition of HIV infection in mDCs when these cells were preincubated with LL-37 prior to HIV exposure (Figure 6A), indicating that LL-37 effects on HIV infectivity are differentially regulated in LCs and DCs. LL-37 did not affect CD4 or A3G expression in DCs but markedly downregulated surface expression of CCR5 and DC-SIGN (Figures 6B and 6C). It has been shown that DC-SIGN binds HIV and plays a critical role for HIV replication in mDCs (Gringhuis et al., 2010). These results, in contrast to mLCs, suggest that decreased HIV infection levels observed in LL-37-treated mDCs may be due to downregulation of DC-SIGN and/or CCR5 on their cell surfaces. Taken together, our results indicated the presence of exclusive machinery to augment HIV infection by LL-37 in LC in contrast to that in $CD4^+$ T cells (Bergman et al., 2007) and mDCs.

### LL-37 Enhances HIV Transmission from LCs to $CD4^+$ T Cells

We next examined whether LL-37 affected HIV transmission from LCs to cocultured $CD4^+$ T cells. mLCs or mDCs were stimulated with AMPs or TNF-α for 24 hr, exposed to $HIV\text{-}1_{Ba\text{-}L}$, and then cocultured with allogeneic $CD4^+$ T cells for 12 days. Consistent with results showing that LL-37 increases HIV infection levels in mLCs (Figure 3A), preincubation of mLCs with LL-37 significantly enhanced subsequent HIV transmission from mLCs to $CD4^+$ T cells in a dose-dependent manner; preincubation with other AMPs did not affect HIV transmission levels in mLC-T cell cocultures (Figure 7A). By contrast, HIV transmission from mDCs to $CD4^+$ T cells was significantly decreased by preincubation of mDCs with LL-37 (Figure 7B), consistent with decreased HIV infection levels in LL-37-treated mDCs (Figure 6A).

## DISCUSSION

LCs are generally believed to be one of the cell types that plays a pivotal role in the dissemination of virus during sexual transmission of HIV. To understand the biologic mechanisms by which HSV-2 increases acquisition of HIV, we tested the hypothesis that HSV-2 modulates LC susceptibility to HIV. As expected, we found that HSV-2 enhances HIV susceptibility of LCs within epithelial tissue (Figure 1), consistent with a recent finding that HSV-2 directly enhances HIV susceptibility in LCs (de Jong et al., 2010). However, in our ex vivo explant model, the percentage of HSV/HIV-coinfected LCs was quite low. Instead, our findings suggested that HSV-2 increases HIV susceptibility in LCs by indirect (i.e., epithelial cell-dependent) mechanisms. More specifically, we show here that LL-37 produced by HSV-2-infected epithelial cells enhances HIV infection of LC, most likely by increasing surface expression of CD4 and CCR5 on these cells.

There are conflicting prior reports on how defensins affect HIV infectivity. A variety of anti-HIV activities for hBD2 and hBD3 have been reported, including direct inhibition of virions, indirect inhibition of HIV replication, and downregulation of HIV coreceptors (Klotman and Chang, 2006; Quiñones-Mateu et al., 2003). By contrast, other studies have shown increased HIV infection of

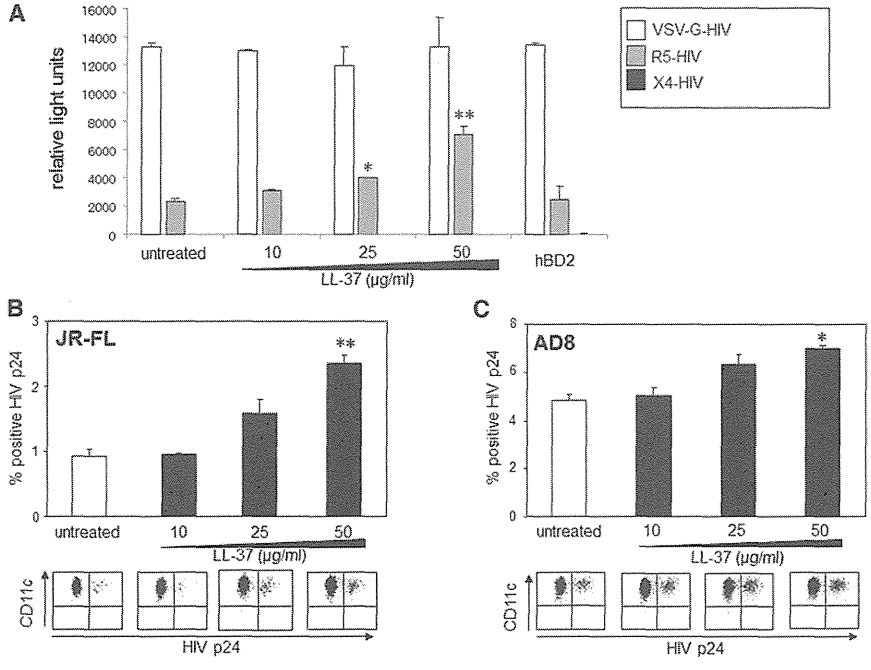


**Figure 5. LL-37 Enhances mLCs Susceptibility to R5-HIV and R5 HIV Primary Isolates**

mLCs were stimulated with LL-37 at the indicated concentration or hBD2 as control, and then exposed to pseudotyped viruses (R5-HIV, X4-HIV or VSV-G-HIV) for 72 hr (A) or R5 HIV primary isolates (JR-FL or AD8) for 2 hr (B and C). To assess pseudotyped virus infection levels, the average luciferase activity was calculated as relative light units (A). To assess primary HIV infection levels, mLCs were collected 7 days after the HIV exposure, and HIV p24$^+$ cells were assessed in langerin$^+$ CD11c$^+$ mLCs (upper panels, % of positive cells for HIV p24 in langerin$^+$ CD11c$^+$ mLCs; and lower panels, representative flow cytometric analyses following LL-37 stimulation). Results are shown as means ± SD (*$p < 0.05$; **$p < 0.01$). All data shown represent at least two separate experiments. See also Figure S5.

primary CD4$^+$ T cells by HD5 and HD6, and no effects on cell-surface HIV coreceptor expression by hBD1 and hBD2 (Klotman et al., 2008; Sun et al., 2005). These conflicting reports might be due to differences in experimental conditions or cell types used (e.g., PBMC or CD4$^+$ T cells). Interestingly, we found that, unlike PBMC and CD4$^+$ T cells, human β defensins did not affect HIV infectivity of LCs (Figure 3). Although no significant differences were detected, hBD2 and HD5 tended to decrease HIV infectivity of LCs. In addition, consistent with a previous finding that LL-37 inhibits HIV replication in CD4$^+$ T cells (Bergman et al., 2007), we found that LL-37 significantly inhibited HIV infectivity in DCs, probably through downregulation of surface DC-SIGN expression (Figure 6). By contrast, LL-37 upregulated surface expression of CD4 and CCR5 in LCs, and upregulation strongly correlated with the increased R5 HIV entry and infection within these cells (Figure 4 and Figure 5). Thus, these findings clearly indicate that the effects of AMPs on HIV infectivity are differentially regulated depending on the target cell type.

We found that HSV-2 infection in epithelial cells induced the production of soluble LL-37 that had potent enhancing effects on HIV infectivity in LCs. siRNA-mediated interference of LL-37 transcription blocked, at least in part, increased HIV infectivity. We hypothesize that when HSV-2 infection occurs in genital mucosa, LL-37 produced by HSV-infected epithelial cells augments HIV susceptibility of LCs, thereby leading to enhanced sexual transmission of HIV. Notably, a recent study has shown that cervicovaginal levels of LL-37 were associated with increased HIV acquisition in Kenyan sex workers (Levinson et al., 2009), a clinical finding that supports our hypothesis. Thus, HSV-2 can mediate both direct enhancing effects on HIV susceptibility in LCs as well as indirect enhancing effects via LL-37 production as shown here. These results further our understanding of the complex biologic events that occur during the early stages of sexual transmission of HIV.

## EXPERIMENTAL PROCEDURES

### Reagents

Cells were stimulated with synthetic AMPs (Peptide Institute) for 24 hr at the following concentrations: α defensin-5 (50 μg/ml), β defensin-1 (50 μg/ml), β defensin-2 (50 μg/ml), β defensin-3 (5 μg/ml), β defensin-4 (50 μg/ml), and LL-37 (50 μg/ml). Recombinant human (rh) TNF-α (5 μg/ml, R&D Systems) was used as a positive control in some experiments. Anti-TNF-α neutralizing mAbs (clone; MABTNF-A5) were purchased from BD PharMingen and used at a final concentration of 1 μg/ml.

### Cell Preparation

NHEKs were purchased from Kurabo and cultured with EpiLife supplemented with insulin (10 μg/ml), rhEGF (epidermal growth factor, 0.1 ng/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), and bovine pituitary extract (0.4% V/V) (EpiLife-KG2 medium, all from Kurabo) in a humidified atmosphere with 5% CO2 at 37°C.

mLCs and mDCs were cultured from adult plastic-adherent PBMCs as described previously (Kawamura et al., 2001). Briefly, monocytes were isolated by depletion of magnetically labeled nonmonocytes (Monocyte Isolation Kit II, Miltenyi Biotec) from plastic-adherent PBMCs obtained from healthy blood donors. Monocytes were cultured in RPMI 1640 (GIBCO BRL) supplemented with 10% heat-inactivated FBS (Cell Culture Technologies), 100 U/ml penicillin (GIBCO BRL), 100 μg/ml streptomycin (GIBCO BRL), 2 mM L-glutamine (GIBCO BRL) (complete medium) supplemented with 1,000 U/ml rhGM-CSF (R&D Systems), 1,000 U/ml rhIL-4 (R&D Systems), and with mLCs or without mDCs 10 ng/ml human platelet-derived TGF-β1 (R&D Systems) for 7 days. Since we have previously found the expression levels of E-cadherin$^+$ cells and langerin$^+$ cells in mLCs to be approximately 90% and 35%, respectively (Kawamura et al., 2001), cell sorting was performed at day 7 to isolate highly purified langerin-positive mLCs followed by staining with anti-langerin mAb (Immunotech), as previously described (Ogawa et al., 2009). Alternatively, mLCs were identified by gating langerin-positive cells in flow cytometric analyses.

### HSV-2 Exposure of Cells In Vitro and Skin Explants Ex Vivo

Purified, pelleted, and titered HSV-2 G strain (stock at $10^8$ PFU/ml) was purchased from Advanced Biotechnologies. HSV-2 strain 186 (stock at $1.5 \times 10^7$ PFU/ml) was a gift from Yukihiro Nishiyama (Nagoya University Graduate School of Medicine, Nagoya, Japan). A total of $2 \times 10^5$ mLCs or mDCs, or $5 \times 10^6$ NHEK, were cultured with different concentrations of HSV-2 ($10^{4-6}$ PFU) at 37°C, and then washed three times. In some experiments using the supernatants from NHEKs treated by HSV-2, culture supernatants

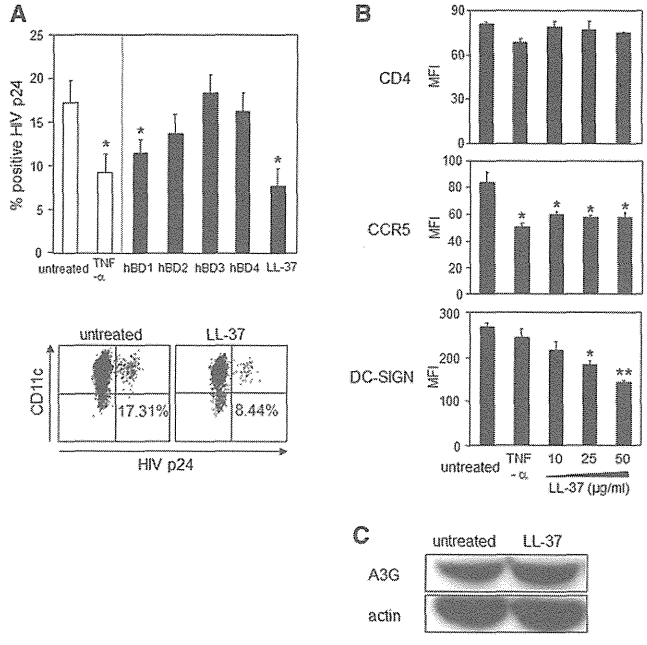


**Figure 6. LL-37 Decreases HIV Susceptibility in mDCs**

(A) mDCs were stimulated with the indicated AMPs or rhTNF-α 24 hr prior to HIV exposure. To determine HIV infection levels, mDCs were collected 7 days after HIV exposure, and HIV $p24^+$ cells were assessed in $CD11c^+$ mDCs. Representative flow cytometric analyses are shown.

(B) mDCs were stimulated with TNF-α or LL-37 at the indicated concentrations for 24 hr. The expression of CD4, CCR5, and DC-SIGN was assessed by flow cytometry.

(C) The expression of A3G was determined by western blot analysis. Results are shown as means ± SD (n = 3) (*$p < 0.05$; **$p < 0.01$). All data shown represent at least two separate experiments.

containing HSV-2 were filtered by PALL Acrodisc 32 mm Syringe Filter with 0.1 μm Supor Membrane to remove viruses. For control infection, the same batch of virus was inactivated at 56°C for 10 min and the same volume as the active virus was added to cells. For exposure of epithelial tissue explants, 50 μl droplets containing different concentrations of HSV-2 were placed on the inside surfaces of sterile plastic culture dish covers. Explants were draped over droplets with the basal epithelial cell surface facing downward. Virus and explants were incubated together in this manner at 37°C in a humidified 5% $CO_2$ environment for 1 hr, and then washed three times with cold PBS.

### HIV Infection of Cells In Vitro and Skin Explants Ex Vivo

Purified, pelleted, and titered HIV-1Ba-L, an R5 HIV laboratory isolate (stock at $TCID_{50}$ of $10^{7.17}$/ml and 1.8 × $10^{10}$ virus particles/ml), was purchased from Advanced Biotechnologies. Molecular clones R5 HIV primary isolates (JR-FL and AD8) were prepared as described previously (Koyanagi et al., 1997; Theodore et al., 1996). Briefly, 293T cells were transfected with 30 μg of HIV-1 proviral DNA. One day after transfection, the medium was replaced with fresh RPMI 1640 medium supplemented with 10% FCS, and then 2 days later, the viruses were recovered, filtered through a membrane (pore size, 0.22 mm), and assayed for HIV-1 p24 gag content by ELISA. The titer of each virus stock was determined by endpoint titer determination of 3-fold limiting dilution in triplicate on PHA-activated PBMC from a single donor. Aliquots of the viral stocks ($TCID_{50}$ of 9,004,929/ml; JR-FL and 7,746,147/ml; AD8) were stored at minus 80°C until use. For some experiments, 2 × $10^5$ mLCs and mDCs were preincubated with various agonists, inhibitors, HSV-2, or the supernatants from NHEKs treated by HSV-2, and then HIV-1Ba-L at a 1/100 final dilution or R5 HIV primary isolates (JR-FL and AD8) at $TCID_{50}$ of $10^5$/ml was added for 2 hr at 37°C, as described previously (Kawamura et al., 2001). After incubation, cells were harvested, washed three times in washing medium (HBSS containing 10% heat-inactivated FBS), resuspended in complete medium supplemented with GM-CSF and IL-4, and cultured for 7 additional days at the same cellular concentration. HIV-infected cells were assessed by HIV p24 intracellular staining. Because the variability in the infection levels was most likely due to the *CCR5* heterogeneity in the donors, HIV infection levels with mLCs and mDCs obtained from different donors were not directly compared. Instead, HIV infection levels were expressed as a normalized percent of the positive cells for HIV p24 by using a calculated fold difference as compared with the mean percent of the positive cells for HIV p24 in untreated cells (Figure 3A). In some experiments, 2 × $10^4$ HIV-infected mLCs or mDCs were cocultured with 2 × $10^6$ allogeneic $CD4^+$ T cells for 12 days, and supernatants were harvested every third day and examined for HIV p24 protein content by ELISA (ZeptoMetrix) according to the manufacturer's instructions.

Epithelial sheets were obtained from suction blister roofs from HIV-negative healthy donors. For infection of epithelial tissue explants, 50 μl droplets containing HIV-1Ba-L at a 1/100 final dilution were placed on the inside surfaces of sterile plastic culture dish covers, as described previously (Kawamura et al., 2000). Explants were draped over droplets with the basal epithelial cell surface facing downward. Virus and explants were incubated together in this manner at 37°C in a humidified 5% $CO_2$ environment for 2 hr. Explants were washed in three separate wells in 6-well plates containing sterile PBS and then floated with the basal epithelial cell sides down in 12-well plates containing 2 ml of complete medium, without exogenous stimulants or cytokines. The emigrating cells from the epidermal sheets were collected 3 days after the HIV exposure. In some experiments, epidermal cell suspensions were prepared by limited trypsinization of epidermal sheets, as described previously (Miller et al., 2011a).

### Pseudotyped Virus Infection and Luciferase Assay

To prepare pseudotyped viruses with Env from either HIV-1 (IIIB, JR-FL, or VSV), 293 T cells were cotransfected with the Env expression plasmid DNA, pLET, pJRFLenv, or pMD.G, respectively, and with pNLLuc (an Env-defective HIV-1NL4-3 carrying the luciferase gene) as described previously (Sato et al., 2008). The culture supernatants were harvested and then filtrated to produce virus solutions at 48 hr posttransfection. To measure the infectivity of Env-pseudotyped virus, mLCs were incubated with JR-FL Env- or IIIB Env-pseudotyped virus, containing 20 ng of p24CA, or VSV envelope glycoprotein-pseudotyped virus, containing 0.5 ng of p24CA, for 72 hr. The Picagene luciferase assay kit (Toyo Ink) was used to perform luciferase assays, following the manufacturer's protocols. Activity was measured with a 1420 ARVOSX multilabel counter (Perkin Elmer) and normalized to the protein content of each lysate, measured with a Coomassie (Bradford) protein assay kit (Pierce).

### Flow Cytometry

Single-cell suspensions were stained using the following anti-human mAb: anti-CD83 (BD Biosciences-PharMingen), anti-CD86 (BD Biosciences-PharMingen), anti-CD4 (Beckman Coulter), anti-CCR5 (R&D), anti-DC-sign (R&D), anti-CCR7 (R&D) directly conjugated to FITC, anti-langerin (Immunotech) directly conjugated to PE, and anti-CD11c (Becton Dickinson) directly conjugated to allophycocyanin. Cells were incubated with Abs for 30 min at 4°C and then washed three times in staining buffer and examined by FACScaliber using propidium iodide (Sigma) to exclude the dead cells in the surface staining.

To specifically identify HIV- or HSV-infected cells on a single-cell level, HIV p24 or HSV gD intracellular staining was performed, respectively. Epidermal LCs, mLCs, and mDCs were collected at the indicated days after HIV exposure and then washed three times in staining buffer, and then incubated with 10 μg/ml allophycocyanin-conjugated mouse anti-human CD11c mAb, and with mLCs or without mDCs PE-conjugated mouse anti-human langerin mAb and for 30 min at 4°C. Cells were then washed three times in staining buffer and fixed and permeabilized with Cytofix/Cytoperm reagents (BD Biosciences-PharMingen) for 20 min at 4°C. Cells were then washed three times in Perm-Wash (BD Biosciences-PharMingen), incubated with FITC- or PE-conjugated mouse anti-HIV p24 mAb (Beckman Coulter) and/or FITC-conjugated mouse anti-HSV gD mAb (Argene) diluted for 30 min at 4°C, and washed three times in Perm-Wash, with the quantified numbers of HIV- or HSV-infected cells determined by FACScaliber.

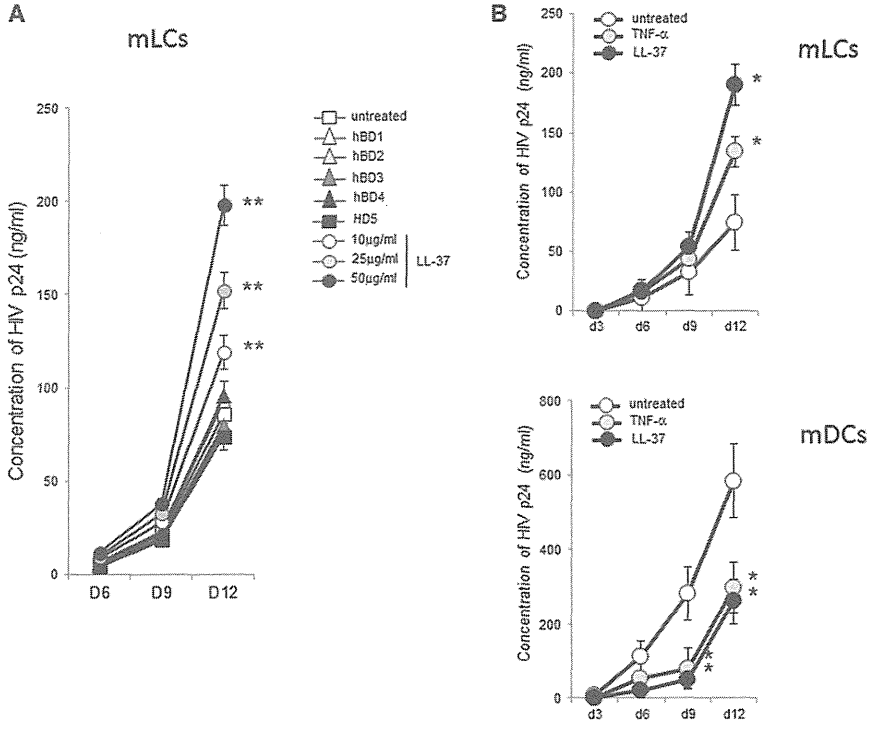


**Figure 7. LL-37 Enhances HIV Transmission from mLCs to T Cells**

mLCs (A) or mLCs and mDCs isolated from the same donor (B) were stimulated with the indicated AMPs or rhTNF-α 24 hr prior to HIV exposure. HIV-infected mLCs or mDCs were cocultured with allogeneic $CD4^+$ T cells, and p24 protein levels in culture supernatants were assessed by ELISA on the indicated days. Results are shown as means plus or minus SD (n = 3). *p < 0.05; **p < 0.01. All data shown represent at least two separate experiments.

### RNA Interference Using siRNA

The delivery of siRNA into NHEKs was performed by DharmaFECT 3 siRNA Transfection Reagent (Dharmacon). Cells were transfected with siRNAs at a final concentration of 50 nM. The siRNAs used in this study were ON-TARGETplus nontargeting pool (Dharmacon #D 001810–10) for control siRNA and ON-TARGETplus SMARTpool siRNA Human CAMP (Dharmacon #L-019790–00) for LL-37 siRNA.

### Real-Time Quantitative RT-PCR Analysis

Relative mRNA expression was determined by real-time PCR using an ABI PRISM 5500 Sequence Detection System (Applied Biosystems) with SYBR Green I dye (QIAGEN) according to the manufacturer's instructions. Total RNA was isolated using TRIzol (Invitrogen Life Technologies), and cDNA was synthesized using the SuperScript system (Invitrogen Life Technologies). Primers corresponding to human α defensin-5, defensin-6, human β defensin-1, β defensin-2, β defensin-3, β defensin-4, LL-37, and GAPDH were designed by Takara Bio, Inc. Cycle threshold numbers (Ct) were derived from the exponential phase of the PCR amplification. Fold differences in the expression of gene x in the cell populations y and z were derived by 2k, where k = (Ctx − CtG3PDH)y − (Ctx − CtG3PDH)z.

### ELISA

NHEKs were exposed to live HSV-2 ($10^6$ PFU) or heat-inactivated HSV-2 for 1 hr, and then washed three times. Following culture in medium for the indicated days, the culture supernatants were collected after centrifugation and stored at −80°C for LL-37 and TNF-α measurement. The concentration of human LL-37 (Hycult biotechnology) and TNF-α (R&D Systems) in the culture supernatants was measured by ELISA. For measurement of HIV p24 protein levels, supernatants were collected, inactivated with Triton X-100 (Sigma-Aldrich; 2% final concentration), and kept frozen until measurements of HIV p24 protein levels were performed by ELISA (ZeptoMetrix).

### Western Blot Analysis

Proteins of the cells were extracted using 15 min incubation in complete lysis buffer containing a protease inhibitor. Equal amounts of protein were separated by SDS-PAGE and transferred onto a transfer membrane (Daiichikagaku). Western blot was performed in order to detect hCAP18 (2.0 μg/ml, Abcam), KLK5 (2.0 μg/ml, R&D Systems), LL-37 (2.0 μg/ml, Santa Cruz), A3G (2.5 μg/ml, Abcam), and SAMHD1 (2.0 μg/ml, Abcam). Blots were incubated with the HRP-linked secondary antibody. Analyses were performed using the HRP western blot detection system (Pierce), and band intensities were calculated using ImageJ software.

### Statistical Analyses

Significant differences between experimental groups were analyzed by Student's t test (one-tailed). p values less than 0.05 were considered significant.

### Study Approval

The Institutional Review Board of the University Hospital (University of Yamanashi, Yamanashi, Japan) approved the acquisition of human tissues, and informed consent was obtained from all skin donors.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article at http://dx.doi.org/10.1016/j.chom.2012.12.002.

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CASE REPORT

# Raltegravir can be used safely in HIV-1-infected patients treated with warfarin

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**Summary:** Drug co-administration often affects the patient response to warfarin through various mechanisms. We describe here five HIV-1-infected patients on treatment with warfarin in whom the use of raltegravir was associated with a favourable outcome.

**Keywords:** HIV/AIDS, warfarin, raltegravir, etravirine, cytochrome P450, drug interaction, antiretroviral therapy

Drug co-administration often affects the patient response to warfarin through various mechanisms. For example, some drugs induce or inhibit liver enzymes, such as cytochrome P450 (CYP) isozymes responsible for warfarin metabolism;[1,2] others alter warfarin sensitivity by changing vitamin K synthesis or absorption, alter warfarin distribution or metabolism by increasing its affinity for receptor sites, or change the synthesis of functional coagulation factors. As the life expectancy of HIV-infected individuals is becoming longer, co-administration of warfarin with antiretrovirals needs to be assessed carefully. Nevirapine and lopinavir–ritonavir reduce serum concentrations of warfarin,[3,4] while efavirenz increases the concentration,[4] probably by the induction and inhibition of CYP2C9,[1,2] the main enzyme in warfarin metabolism. We reported previously the favourable effects of non-boosted fosamprenavir in patients treated with warfarin.[5] The clinical use of warfarin co-administered with raltegravir has not been described so far, though raltegravir seems to be a safe choice because it does not inhibit or induce CYP isoenzymes.[6] We describe here five HIV-1-infected patients on treatment with warfarin in whom the use of raltegravir was associated with a favourable outcome (Table 1). Cases 1–3 were Japanese men who had been treated with a stable dose of warfarin (mean daily dose, 3–4 mg) for underlying diseases, and their international normalized ratios (INR) were maintained within the optimal ranges (1.5–2.5 or 2.0–3.0) before the introduction of antiretroviral therapy (ART). Dose modification of warfarin was not necessary after starting ART containing raltegravir, as INRs remained within the optimal ranges. Case 4 was a 62-year-old Japanese man who had been treated with abacavir, lamivudine and non-boosted fosamprenavir (1400 mg twice daily). Based on his request, ART was switched to abacavir, lamivudine and raltegravir, and INR was maintained within the optimal range (1.5–2.5). Therefore, warfarin dose modification was not necessary. Case 5 was a 57-year-old Japanese man who had been treated with abacavir, lamivudine and lopinavir/ritonavir. He developed chronic atrial flutter. The initial dose of warfarin was 1 mg/day to maintain INR within the optimal range (1.5–2.5). Three months later, INR control became difficult at 4 mg/day of warfarin (INR; 0.70–0.91) and warfarin was terminated because it seemed ineffective. Non-boosted fosamprenavir could not be used because genotypic analysis showed resistance of HIV-1 to fosamprenavir. When raltegravir became available in Japan (9 months after discontinuation of warfarin), treatment was switched to ART comprising abacavir, lamivudine, raltegravir and etravirine, as well as warfarin (at initial dose of 1 mg/day). Three months later, INR was controlled within 1.46–2.49 at 3.5 mg of warfarin. The new regimen allowed maintenance of INR within the optimal range.

Cardiovascular events are increasing with the long-term use of ART. For patients treated with warfarin, raltegravir is a safe and clinically effective ART agent. Etravirine can potentially interact with warfarin by inducing CYP3A and mild inhibition of CYP2C9 and CYP2C19.[7] However, in Case 5, it was used successfully in combination with raltegravir. Such combinations may be helpful for the control of drug-resistant HIV-1 in warfarin-treated patients. Genetic polymorphisms in CYP2C9 may affect the response to warfarin,[8] though such data were not available in our five patients. The clinical introduction of raltegravir has expanded the ART options, though further clinical evidence is necessary in warfarin-treated patients.

**Conflict of interest:** None declared.

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Table 1 HIV-1 infected patients with favourable outcome following treatment with raltegravir and warfarin

| No. | Age (years) | Sex | Underlying disease | ART regimen | Dose of warfarin (mean daily dose) (mg) | Maintained INR | Follow-up period (months) | Remarks |
|---|---|---|---|---|---|---|---|---|
| 1 | 55 | M | Chronic atrial flutter, cerebral embolism | RAL TDF FTC | 3.5–4 | 1.69–2.64 | 15 | – |
| 2 | 57 | M | Portal vein thrombosis | RAL ABC 3TC | 3 | 1.68–2.31 | 2 | – |
| 3 | 59 | M | Chronic atrial flutter, cerebral embolism | RAL ABC 3TC | 3 | 2.03–2.94 | 3 | |
| 4 | 62 | M | Chronic atrial flutter | RAL ABC 3TC | 2.0–2.5 | 1.46–2.49 | 5 | Switched non-boosted FPV to RAL. |
| 5 | 57 | M | Chronic atrial flutter | RAL ETV ABC 3TC | 3.5 | 1.60–1.71 | 5 | Switched LPV/RTV to RAL/ ETV |

RAL = raltegravir 800 mg/day; ETV = etravirine 400 mg/day; TDF = tenofovir 300 mg/day; FTC = emtricitabine 200 mg/day; ABC = abacavir 600 mg/day; 3TC = lamivudine 300 mg/day; FPV = fosamprenavir 2800 mg/day; LPV/RTV = lopinavir 800 mg/day and ritonavir 200 mg/day

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