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Calcitonin gene–related peptide inhibits Langerhans cell–mediated HIV-1 transmission

Yonatan Ganor,1,2,3 Anne-Sophie Drillet-Dangeard,1,2,3 Lucia Lopalco,4 Daniela Tudor,1,2,3 Giuseppe Tambussi,4 Nicolas Barry Delongchamps,5 Marc Zerbib,5 and Morgane Bomsel1,2,3

Upon its mucosal entry, human immunodeficiency virus type 1 (HIV-1) is internalized by Langerhans cells (LCs) in stratified epithelia and transferred locally to T cells. In such epithelia, LCs are in direct contact with peripheral neurons secreting calcitonin gene–related peptide (CGRP). Although CGRP has immunomodulatory effects on LC functions, its potential influence on the interactions between LCs and HIV-1 is unknown. We show that CGRP acts via its receptor expressed by LCs and interferes with multiple steps of LC-mediated HIV-1 transmission. CGRP increases langerin expression, decreases selected integrins, and activates NF-κB, resulting in decreased HIV-1 intracellular content, limited formation of LC–T cell conjugates, and elevated secretion of the CCR5–binding chemokine CCL3/MIP-1α. These mechanisms cooperate to efficiently inhibit HIV-1 transfer from LCs to T cells and T cell infection. In vivo, HIV-1 infection decreases CGRP plasma levels in both vaginally SHIV–challenged macaques and HIV-1–infected individuals. CGRP plasma levels return to baseline after highly active antiretroviral therapy. Our results reveal a novel path by which a peripheral neuropeptide acts at the molecular and cellular levels to limit mucosal HIV-1 transmission and suggest that CGRP receptor agonists might be used therapeutically against HIV-1.
Figure 1. **CGRP inhibits HIV-1 transfer from LCs to T cells.** (A and B) MDLCs were left untreated or pretreated for 24 h with 100 nM CGRP. The cells were then pulsed with $10^1$–$10^3$ (A) or $10^3$ (B) TCID$_{50}$ HIV-1 and co-cultured with autologous CD4$^+$ T cells for 1 wk. Untreated MDLCs were also co-cultured with T cells in the presence of AZT or cultured alone (A, open bar). In A, HIV-1 replication was measured in the culture supernatants by p24 ELISA, and results represent mean ± SEM ng/ml p24 from duplicate wells of a representative experiment of $n = 9$; *, $P < 0.0001$; ANOVA. In B, the cells were collected at the end of the co-culture period, stained with CD3-APC (surface) and p24-PE (intracellular) mAbs, and examined by flow cytometry; shown are...
the calcitonin receptor with RAMP1-3), which mediate the previously described CGRP type 2 receptor phenotype (Poyner et al., 2002).

CGRP appears as a possible modulator of LC function. CGRP neurons are in direct contact with LCs in the skin, and early observations showed that CGRP inhibits LC antigen presentation to T cells (Hosoi et al., 1993). A later study demonstrated that although CGRP inhibits LC-mediated Th1 antigen presentation and cytokine secretion, it enhanced that of Th2 (Ding et al., 2008). Herein, we hypothesized that CGRP could also interfere with the interactions between LCs and HIV-1. As peripheral neurons are lost upon tissue sampling, potential interactions were never studied at the mucosal level. Our results show that CGRP affects multiple molecular and cellular events in LCs, resulting in efficient inhibition of HIV-1 transfer from LCs to T cells and T cell infection.

RESULTS AND DISCUSSION

HIV-1 transfer from LCs to T cells

To measure the transfer of HIV-1 from LCs to T cells, we prepared blood monocyte-derived LCs (MDLCs) and pulsed the cells with the HIV-1 molecular clone JRCSF (clade B, R5 tropism). MDLCs were then co-cultured with autologous CD4+ T cells, and HIV-1 replication was measured in the co-culture supernatants 1 wk later by p24 ELISA. In line with previous observations (de Witte et al., 2007), MDLCs inefficiently transferred HIV-1 to T cells at low viral concentrations (Fig. 1 A), corresponding to 10^1 and 10^2 tissue culture infectious doses (TCID_{50}). In contrast, at a high HIV-1 concentration of 10^3 TCID_{50}, MDLCs efficiently transferred the virus to T cells, a process which was significantly abrogated by the antiretroviral drug azidothymidine (AZT; Fig. 1 A). MDLCs pulsed with 10^3 TCID_{50} HIV-1 and cultured alone without T cells inefficiently replicated the virus (Fig. 1 A).

To confirm these results using a direct read-out for viral replication, the cells were collected at the end of the co-culture period, double-stained for surface CD3 and intracellular p24, and examined by flow cytometry. A clear population of CD3+p24+ infected T cells was detected, which was completely absent when AZT was included during the co-culture period (Fig. 1 B; mean ± SEM percentages of CD3^+p24^ infected T cells derived from n = 5 experiments of 7.4 ± 0.7% and 0.3 ± 0.1%, respectively; P < 0.0001). In contrast, when the cells were double-stained for surface CD1a and intracellular p24, a significantly lower proportion of CD1a+ cells was p24+ (1.3 ± 0.2%, n = 5; P < 0.0001 vs. CD3^+p24^ cells), confirming the inefficient replication of HIV-1 in MDLCs. These results show that our experimental settings are appropriate for the investigation of HIV-1 transfer from LCs to T cells.

MDLCs were next pretreated with CGRP at a concentration range previously found effective in suppressing LC-mediated antigen presentation (0.1–100 nM; Hosoi et al., 1993), before the HIV-1 pulse, and HIV-1 transfer to T cells and T cell infection were measured as described above. Because the efficiency of viral transfer varied when using MDLCs and T cells prepared from different human individuals, the calculated p24 levels in the co-culture supernatants using untreated MDLCs were normalized to 100% for each experiment, allowing for direct comparison. CGRP pretreatment of MDLCs resulted in a dose- and time-dependent inhibition of HIV-1 transfer to T cells, reaching maximal inhibition of 73.3 ± 4.2% after pretreatment with 100 nM CGRP for 24 h (Fig. 1 A–C). Similar inhibition was observed when MDLCs were pulsed with another clade B/R5 HIV-1 molecular clone (ADA; not depicted). The inhibitory effect of CGRP was not a result of increased mortality, as the viability of both untreated and CGRP-treated MDLCs at the end of the co-culture period was similar (~90% as evaluated by trypan blue exclusion).

Although CGRP modulates several T cell functions (e.g., adhesion and cytokine secretion), HIV-1 transfer was not affected when T cells were pretreated with CGRP and then co-cultured with untreated but HIV-1–pulsed MDLCs (Fig. 1 C). Previous observations also showed that CGRP inhibits antigen presentation to T cells when LCs, but not T cells, are pretreated with CGRP (Hosoi et al., 1993).

The inhibitory effect of CGRP was mediated specifically via the CGRP receptor, as the CGRP receptor antagonist CGRP_{8-37} abrogated this effect (Fig. 1 C), whereas the linear receptor agonist Cys(Acm)_{2,7} that acts on related amylin receptors, which are also activated by CGRP (Poyner et al., 2002), was ineffective (Fig. 1 C). In agreement with a potential role of this receptor, RT-PCR showed that MDLCs expressed representative FACS plots, with numbers indicating the mean ± SEM percentages of CD3^+p24^ infected T cells of n = 5 experiments. (C) MDLCs were left untreated or pretreated, as indicated, for 3–24 h with 0.1–100 nM CGRP, 1,000 nM CGRP_{8-37} (added alone or 10 min before addition of CGRP), or 100 nM Cys(Acm)_{2,7}. The cells were then pulsed with 10^3 TCID_{50} HIV-1 and co-cultured with autologous CD4+ T cells for 1 wk. In some experiments, T cells were pretreated with CGRP and then co-cultured with untreated but HIV-1–pulsed MDLCs (gray bar). HIV-1 replication was measured in the co-culture supernatants by p24 ELISA, and results represent mean ± SEM from n = 9 experiments of HIV-1 transfer normalized against untreated cells serving as the 100% set point; numbers represent percentage of HIV-1 transfer inhibition; *, P = 0.0220 and 0.0003 and P < 0.0001 and 0.0001 for 1 nM/3 h, 10 nM/3 h, 100 nM/3 h, and 100 nM/24 h CGRP-treated versus untreated MDLCs, respectively. (D) RT-PCR images for CRLR, RAMP1-3, and RCP in MDLCs from three individuals with total human brain (HB) RNA serving as positive control. (E and F) MULCs were left untreated or pretreated for 24 h with 0.1–100 nM CGRP, culture supernatants of T cells containing 70 nM (high) or 4 nM (low) CGRP (F, striped bars), 1,000 nM CGRP_{8-37}, (added alone or 10 min before addition of CGRP), or 100 nM Cys(Acm)_{2,7}, as indicated. The cells were then pulsed with 10^3 TCID_{50} HIV-1 and co-cultured with CD4+ T cells for 1 wk. HIV-1 replication was measured in the co-culture supernatants by p24 ELISA, and results of n = 5 experiments are presented as in A and B, respectively; p24 level of untreated MULCs cultured alone was 12.0 ± 0.2 ng/ml; *, P = 0.0315 for MULCs pretreated with 100 nM CGRP versus untreated MULCs (E); *, P = 0.0008 and 0.0338 for MULCs pretreated with 100 nM CGRP and high CGRP versus untreated MULCs, respectively (F). (G) RT-PCR images for CRLR, RAMP1-3, and RCP in MULCs (representative of n = 3 experiments).
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Inhibitory effects of both synthetic and endogenous CGRP were mediated via the CGRP receptor, as they were abrogated by CGRP8–37, whereas Cys(Acm)2,7 was ineffective (Fig. 1 F). MULCs expressed CRLR, RAMP1, RAMP2, and RCP, but not RAMP3, as shown by RT-PCR (Fig. 1 G). Expression of CRLR and RAMP1 was lower in MULCs relative to MDLCs. Such reduced expression of CRLR and RAMP1, the later necessary for the targeting of CRLR to the plasma membrane (Walker et al., 2010), might provide a possible explanation for the lower ability of CGRP to inhibit HIV-1 transfer to T cells from MULCs versus MDLCs.

Figure 2. CGRP increases langerin surface expression and decreases HIV-1 replication. (A) Representative FACS histograms showing surface expression of CD1a, CD11c, DC-SIGN, and langerin in untreated and CGRP-pretreated MULCs or LCs derived from inner foreskin versus matched isotype controls. Numbers represent mean ± SEM ratios of the MFI for langerin expression (MFI langerin/MFI isotype) derived from n = 15 (MDLCs) or 5 (foreskin LCs) experiments; P < 0.0001 and 0.0352 for CGRP-treated versus untreated LCs, respectively. (B) MDDCs were left untreated or pretreated for 24 h with 100 nM CGRP, pulsed with HIV-1 at 10^3 TCID_{50}, and either co-cultured with autologous CD4+ T cells or cultured alone (open bar) for 1 wk. HIV-1 replication was measured in the culture supernatants by p24 ELISA. Results represent mean ± SEM ng/ml p24 from n = 4 experiments; *, P = 0.0361 MDDCs alone versus MDDCs + T cells. (C–E) MDLCs were pretreated with CGRP (100 nM, 24 h) alone or after addition of 1,000 nM CGRP8–37 and pulsed with HIV-1 at 10^3 TCID_{50}. MDLCs were then either incubated with trypsin and lysed immediately, and HIV-1 was measured in the cell lysates (C), or cultured alone in the absence of CD4+ T cells, and HIV-1 was measured in the culture supernatants 1 wk later (D). Untreated MDLCs were also pulsed with HIV-1 at 10^3 TCID_{50} in the presence of dynasore or cytochalasin D and co-cultured with autologous CD4+ T cells, and HIV-1 was measured in the co-culture supernatants 1 wk later (E). Results represent mean ± SEM LC intracellular content of HIV-1 (C; pg p24), HIV-1 replication (D; ng/ml p24), or normalized HIV-1 transfer (E; percentage) derived from n = 7, 4, and 3 independent experiments, respectively; numbers in E represent percent inhibition; *, P = 0.0054 and 0.0006 for 100 nM versus no CGRP (C and D); *, P = 0.0145 and 0.0102 for dynasore and cytochalasin D versus untreated MDLCs, respectively (E).
In contrast, CGRP pretreatment (100 nM, 24 h) significantly increased by approximately twofold langerin expression on MDLCs (Fig. 2 A). To substantiate these observations MDDCs to T cells (Fig. 2 B). These results suggest that CGRP-mediated inhibition of HIV-1 transfer does not involve DC-SIGN.

In contrast, CGRP pretreatment (100 nM, 24 h) significantly increased by approximately twofold langerin expression on MDLCs (Fig. 2 A). To substantiate these observations...
Whether CGRP-mediated inhibition of HIV-1 transfer involves modulation of LCs’ adhesive potential, we evaluated the surface expression levels of several integrins. CGRP pretreatment (100 nM, 24 h) of MDLCs decreased the expression of CD29 (β1), CD49d (α5), and CD50 (ICAM-3), while not affecting that of CD18 (β2), CD49f (α6), and CD54 (ICAM-1) integrins (Fig. 3 A). The inhibitory effect of CGRP was mediated via the CGRP receptor, as it was abrogated by CGRP\(_{8-37}\), whereas Cys(Acm)\(_{2-7}\) was ineffective (Fig. 3 A). Consequently, CGRP pretreatment decreased the percentage of MDLCs adhering to fibronectin-coated plates (Fig. 3 B), a process which is mediated by the B1α5 integrin. CGRP also decreased the percentage of conjugates forming between fluorescently labeled MDLCs and T cells, as did antibodies (Abs) to either ICAM-3 or ICAM-1 (Fig. 3 C). Similar results were obtained when conjugate formation was evaluated with MULCs (not depicted).

Our recent study demonstrated that exposure of human adult foreskin explants to HIV-1–infected cells results in increased formation of LC–T cell conjugates (Zhou et al., 2011). However, upon mucosal tissue sampling, peripheral neurons are sectioned and the role of endogenous neuroptides could not be studied. Therefore, we further evaluated the effect of CGRP on LC–T cell conjugate formation and T cell infection in these explants. Direct HIV-1 transfer experiments, as described above, could not be performed with LCs from foreskin explants because of limiting cell numbers. In agreement with our recent findings (Zhou et al., 2011), exposure of inner foreskin explants to HIV-1–infected cells increased the percentage of LC–T cell conjugates compared with exposure to noninfected cells (Fig. 3 D, top; mean ± SEM fold percentage LC–T cell conjugates \([n = 3]\) of 2.1 ± 0.1, \(P = 0.0019\); Student’s \(t\) test). Moreover, a small but significant proportion of T cells was infected in these explants (Fig. 3 D, bottom; \(P = 0.0110\) for HIV-1–infected vs. noninfected cells \([n = 3]\); Student’s \(t\) test). In contrast, CGRP pretreatment (100 nM, 24 h) abrogated the increase in LC–T cell conjugate formation and T cell infection, mediated by HIV-1–infected cells. Both remained similar to that in explants exposed to noninfected cells (Fig. 3 D).

Of note, the effective concentration of 100 nM CGRP used herein in foreskin explants seems to be within its normal range in the skin (e.g., 10–23 pmol CGRP per gram of mouse skin [Ahmed et al., 1998], which translates to 28–65 nM CGRP, considering that skin contains around 35% water). Together, these results suggest that both in vitro and ex vivo, CGRP restricts T cell infection by decreasing LC–T cell conjugate formation.

**CCL3/MIP-1α secretion**

CGRP decreases Th1 and increases Th2 cytokine/chemokine secretion by LCs (Ding et al., 2008). Of special interest in the context of HIV-1 are the CCR5-binding chemokines CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES, which block HIV-1 infection by binding the HIV-1 coreceptor CCR5 on target cells. To investigate whether CGRP-mediated inhibition of HIV-1 transfer might be related to altered secretion...
of these chemokines, we measured their levels comparatively in culture supernatants of CGRP-treated versus untreated MDLCs and MULCs that were pulsed with HIV-1 and cocultured for 1 wk with T cells, as described above. These experiments revealed that pretreatment with 100 nM CGRP resulted in increased CCL3/MIP-1α secretion but did not affect that of CCL4/MIP-1β and CCL5/RANTES (mean ± SEM fold secretion from n = 4 experiments of 1.69 ± 0.1 [P = 0.0003; Student’s t test], 0.9 ± 0.2, and 1.2 ± 0.2, respectively). To test whether CGRP-induced elevation of CCL3/MIP-1α secretion contributes to the inhibition of HIV-1 transfer, we added a CCL3/MIP-1α neutralizing Ab during the co-culture period. The CCL3/MIP-1α neutralizing Ab abrogated CGRP-mediated inhibition of HIV-1 transfer from MDLCs to T cells in a dose-dependent manner, whereas a control Ab had no significant effect (Fig. 3 E). Both the control and CCL3/MIP-1α neutralizing Abs had no effect on HIV-1 transfer from untreated MDLCs to T cells (not depicted). In addition, we also included a CCR5-neutralizing Ab (clone 2D7) in other HIV-1 transfer experiments and found that this Ab completely inhibits viral transfer (not depicted). Together, these results clearly show that blocking CCR5 on T cells by either CCL3/MIP-1α or a neutralizing Ab impairs HIV-1 transfer.

As transcriptional up-regulation of the CCL3/MIP-1α gene is mediated by NF-κB, we hypothesized that CGRP-mediated increase in CCL3/MIP-1α secretion results from NF-κB activation. To test this assumption, we treated MDLCs with 100 nM CGRP alone or in the presence of the pharmacological NF-κB inhibitors BAY117082 or MG132 and evaluated viral transfer to T cells as before. MDLCs were also treated with CGRP in the presence of the PKA inhibitor H89, as CGRP also induces PKA activation upon binding to its receptor (Walker et al., 2010). BAY117082 and MG132 abrogated the inhibition of HIV-1 transfer (Fig. 3 F). This abrogation was accompanied by a reduction in CGRP-induced elevation in CCL3/MIP-1α secretion that returned to baseline levels (mean ± SEM fold secretion from n = 4 experiments of 1.0 ± 0.1 for the highest concentration of BAY117082). In contrast, H89 had no effect on HIV-1 transfer from MDLCs to T cells (Fig. 3 F). These results demonstrate that the anti–HIV-1 activity of CGRP in LCs involves specifically the NF-κB, rather than PKA, signaling cascade.

CGRP plasma levels

HIV-1 infection is associated in vivo with loss of cutaneous innervation and reduced epidermal nerve fiber density (McCarthy et al., 1995; Zhou et al., 2007). As sensory peripheral nerves innervating the skin/foreskin are reactive for CGRP (Hosoi et al., 1993), we hypothesized that their loss expected to occur upon HIV-1 infection in vivo will result in reduction of CGRP levels. We tested this hypothesis by measuring CGRP in plasma of HIV-1–infected individuals, as foreskin tissues from such patients are difficult to obtain. Although the normal plasma level of CGRP is low (picomolar range) and seems insufficient to operate at the mucosal level (nanomolar range), it serves as a clinical peripheral marker for the deregulation of CGRP in several pathological conditions (e.g., increased in patients with migraine and decreased in patients with hypertension). We found that CGRP levels were significantly decreased in a group of HIV-1–infected patients compared with healthy individuals (Fig. 4 A). In an additional group of HIV-1–infected patients receiving highly active antiretroviral therapy (HAART), CGRP levels were normalized back to baseline levels (Fig. 4 A). To determine whether the reduced levels of plasma CGRP in HIV-1–infected individuals result directly from the presence of the incoming virus early in infection, we also measured CGRP levels in female macaques that were vaginally challenged with SHIV to mimic viral transmission during sexual intercourse. These animals were described in our recent study, which demonstrated the protective efficacy of a gp41-based vaccine active at mucosal sites in protecting against repeated vaginal SHIV challenge (Bomsel et al., 2011). Hence, CGRP plasma levels were measured comparatively in the placebo-vaccinated animals before SHIV challenge and at several later time points at which all animals were infected (see Bomsel et al. [2011] for details regarding the immunization protocol, SHIV doses delivered, and viral loads). This analysis showed that CGRP levels gradually decreased after repeated vaginal SHIV challenges (Fig. 4 B). These results suggest that HIV-1/SHIV infection is associated with reduced circulating levels of CGRP in vivo.

We show herein that CGRP efficiently inhibits HIV-1 transfer from LCs to T cells, limits HIV-1 infection of T cells, and is decreased in HIV-1–infected patients and SHIV-challenged
animals. CGRP exerts its anti-HIV-1 activities by a combination of several different mechanisms, rather than by a single distinct one, which cooperate together. As such, CGRP possesses some of the properties that characterize cellular HIV-1 restriction factors (Blanco-Melo et al., 2012): CGRP acts autonomously and exhibits antiviral activity in vitro, it employs unique mechanisms to inhibit viral transfer, and its activity might be counteracted in the presence of HIV-1. Yet, in contrast to cellular HIV-1 restriction factors, CGRP exerts its antiviral potential indirectly. We suggest, therefore, that CGRP could be considered a novel indirect HIV-1-limiting factor, which acts at the extracellular rather than intracellular level.

Activation of the CGRP receptor results in Gsα-mediated activation of adenylyl cyclase, with a subsequent increase in cAMP and activation of PKA (Walker et al., 2010), which results in relaxation of smooth muscle cells and vasodilatation. Interestingly, a previous study showed that although CGRP increases cAMP production in LCs, such increase alone was not sufficient for inhibiting LC-mediated antigen presentation (Ashina et al., 1995). A later study showed that the inhibition of LC-mediated antigen presentation by CGRP involves the NF-κB pathway (Ding et al., 2007). These findings suggest that different signaling pathways contribute to CGRP-induced vasodilatation in smooth muscle cells versus immunosuppression in LCs. Accordingly, our results show that the NF-κB pathway activated by CGRP mediates HIV-1 transfer inhibition.

CGRP is secreted from peripheral neurons that innervate essentially all organs in the body, including mucosal epithelia. These CGRP neurons are in direct contact with LCs in the skin (Hosoi et al., 1993), as well as with macrophages in the skin and lymphoid organs. CGRP inhibits antigen presentation and cytokine secretion not only in LCs but also in macrophages (Tori et al., 1997). We can therefore speculate that the HIV-1 inhibitory activity of CGRP may be relevant also to later time points during HIV-1 infection after LC migration and viral dissemination in such organs. Additionally, CGRP would potentially control HIV-1 spread within the nervous system itself, where macrophages (as well as microglia) are the principal cells targeted by HIV-1.

CGRP is involved in migraine pathophysiology, in which its plasma levels are increased. Clinical trials have clearly demonstrated the efficacy of CGRP receptor antagonists for the treatment of migraine (Olesen et al., 2004). Based on our results herein, we suggest that enhancing CGRP receptor activation in LCs might provide novel means for limiting HIV-1 spread. Hence, novel CGRP receptor agonists, active especially at mucosal epithelia at targeting LCs, might represent a completely new class of anti–HIV-1 molecules.

Our study not only reveals a novel immunosuppressive effect of CGRP on LC function and its mechanisms of action, but also provides for the first time the evidence that the nervous system can restrict “at a distance” the early events of HIV-1 transmission at the mucosal level. Whether CGRP serves as a natural barrier to HIV-1 transmission and contributes to the rather low degree of infection during sexual intercourse remains an open question.

### MATERIALS AND METHODS

**Cells.** PBMCs from healthy donors were separated from whole blood by a standard Ficoll gradient. CD14+ monocytes and CD4+ T cells were obtained from PBMCs by negative magnetic selection (STEMCELL Technologies) according to the manufacturer’s instructions. MUTZ-3 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), and TT cells were obtained from the ATCC. Both cell lines were maintained according to the supplier’s guidelines. MDLCs, MDDCs, and MULCs were prepared and maintained as described previously (Gensmann et al., 1998; Masterson et al., 2002); mean ± SEM percentage of MDLCs expressing surface langerin (see below) was 13.6 ± 4.1% (n = 24).

**HIV-1 transfer.** 105 MDLCs, 105 MDDCs, and 2.5 × 105 MULCs were pretreated with CGRP, CGRP$_{8-37}$ (Sigma-Aldrich), Cys(Acm)$_{5}$ (Bachem), or CGRP-containing TT culture supernatants in a 96-well round-bottom plate (200 µl/well final), washed, and pulsed with HIV-1 JRCSF (National Institutes of Health [NIH]) for 2 h (0.1–10 ng corresponding to 10$^{-3}$–10$^{-1}$ TCID$_{50}$) prepared as described previously (Ganor et al., 2010; Zhou et al., 2011). In some experiments (for example, those with CGRP) was added during the pulse period at the indicated concentrations. The cells were then incubated with trypsin for 10 min and lysed with NP-40 or washed again and incubated with T cells (3 × 10$^{5}$; autologous for MDLCs). In other experiments, AZT (Sigma-Aldrich), normal goat IgG, neutralizing CCL3/MIP-1α goat Ab (R&D Systems), the NF-κB inhibitors BAY117082 and MG132 (Santa Cruz Biotechnology, Inc.), or the PKA inhibitor H89 (Tocris Bioscience) was included. HIV-1 was measured by p24 ELISA (Innotest; Innogenetics) immediately in the cell lysates or in the co-culture supernatants 1 wk later. The cells were also collected at the end of the co-culture period, double labeled for surface CD3 or CD1a and intracellular p24 expression, and examined by flow cytometry as we recently described (Ganor et al., 2013).

**RT-PCR.** Expression of components comprising the CGRP and related receptors was evaluated by RT-PCR as described previously (CGR and RAMP2 [Sams and Jansen-Olesen, 1998]; RAMP1 and RAMP3 [Bailey and Hay, 2006], and RCP [Moreno et al., 2002]); total human brain RNA (Takara Bio Inc.) served as positive control.

**Flow cytometry.** MDLCs (10$^{5}$/well) were stained with APC-conjugated mouse anti-human CD1a, CD11c, and DC-SIGN mAbs (BD), PE-conjugated mouse anti–human langerin mAb (clone DCGM4; Beckman Coulter), or 10 µg/ml of the indicated primary mouse anti–human integrin mAbs (ImmunoTools) followed by 1:50 dilution of a secondary FITC-conjugated anti–mouse IgG (Jackson ImmunoResearch Laboratories, Inc.). Cells stained with matched isotype mAbs served as negative control. Each step was performed for 30 min on ice at 50 µl/well. Fluorescent profiles were recorded using a FACSCalibur (BD), and results were analyzed using the CellQuest Pro software (BD).

**Adhesive potential and inner foreskin explants.** Adhesion in vitro to fibronectin (Sigma-Aldrich)-coated plates (5,000 MDLCs/well) was performed as we described previously (Ganor et al., 2007). For conjugate formation in vitro, 10$^{5}$ MDLCs were labeled according to the manufacturer’s instructions with CellTracker green (Molecular Probes), pretreated with CGRP, washed, and incubated with 3 × 10$^{5}$ CellTracker orange–labeled T cells for 1 h. Untreated and labeled MDLCs were also incubated with labeled T cells in the presence of mouse anti–human CD50 (ICAM-3) or CD54 (ICAM-1) mAbs (ImmunoTools) at 20 µg/ml. The percentage of double-positive conjugates was measured by flow cytometry. Inner foreskin explants were prepared, inoculated with HIV-1–infected PBMCs, and processed for flow cytometry as we recently described (Zhou et al., 2011).

**Ethical approval and human study population.** All human participants were recruited at the Department of Infectious Diseases of the San Raffaele Scientific Institute, Milan, Italy. The institutional review board and the local...
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ethical committee of the San Raffaele Scientific Institute, the Comitato Etico dell'Istituto Scientifico Ospedale San Raffaele, approved the investigations. All human subjects provided informed consents. Animals from which blood was sampled were maintained under guidelines established by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals, as we described previously (Bomsel et al., 2011).

A total of 38 plasma samples were selected for the study as follows: (a) 14 samples from healthy individuals not exposed to HIV-1; (b) 14 samples from treatment-naive HIV-1–infected individuals, including seven primary and seven chronic HIV-1–infected patients with mean (and range) of CD4+ T cell count of 330 (149–495)/µl and HIV-1 RNA 1,154,073 (5,169–11,000,000) copies/ml. Primary HIV-1 infection was defined on the basis of the presence of an acute clinical syndrome, a positive test for HIV-1 RNA in plasma, and the presence of less than three positive bands in a Western blot assay; (c) 10 samples from HAART-treated HIV-1–infected patients with mean (and range) of CD4+ T cell count of 738 (317–2,184)/µl, on antiretroviral treatment for at least 24 and not more than 30 mo with chronic and progressive infection, but without previous AIDS defining disease. Samples were matched for sex, age, and risk factors.

Measurement of chemokines and plasma CGRP. Chemokines levels were measured using a multiplex assay and flow cytometry (Bender MedSystems), as we recently described (Zhou et al., 2011). An Enzyme Immuno-Assay (EIA; Bachem) was used to measure human CGRP plasma levels. A similar ELISA (CUSABIO Biotech) was used to measure CGRP plasma levels in six female Macaca mulatta placebo-vaccinated animals, and before and at days 46, 72, 100, and 128 (when all animals were infected) after repeated SHIV challenge, as we recently described (Bomsel et al., 2011).

Statistical analysis. Statistical significance was analyzed by Student’s t-test. For comparing human CGRP plasma levels, pairwise comparisons were performed by the nonparametric Mann–Whitney test.

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Y. Ganor, Y. Zhou, J. Bodo, D. Tudor, J. Leibovitch, D. Mathez, A. Schmitt, M.C. Vacher-Lavenu, M. Revol, and M. Bomsel. 2013. The adult penile urethra is a novel entry site for HIV-1 that preferentially targets resident urethral macrophages. Mucosal Immunol. 6:776–786. http://dx.doi.org/10.1038/mi.2012.116

Geijtenbeek, T.B., D.S. Kwon, R. Toresma, S.J. van Vliet, G.C. van Duijnhooven, J. Middel, I.L. Cornelissen, H.S. Nottet, V.N. KevolRamani, D.R. Littman, et al. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell. 100:587–597. http://dx.doi.org/10.1016/S0028-3908(00)00694-7

Geissmann, F., C. Prost, J.P. Monnet, M. Dy, N. Brousse, and O. Hermine. 1998. Transforming growth factor β1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. J. Exp. Med. 187:961–966. http://dx.doi.org/10.1084/jem.187.6.961

Griffiths, C.E., D. Railan, W.M. Gallatin, and K.D. Cooper. 1995. The ICAM-3/αLFA-1 interaction is critical for epidermal Langerhans cell alloantigen presentation to CD4+ T cells. Br. J. Dermatol. 133:823–829. http://dx.doi.org/10.1111/j.1365-2133.1995.tb06911.x

Hosoi, J., G.F. Murphy, C.I. Egan, E.A. Lerner, S. Grabe, A. Asahina, and R.D. Granstein. 1993. Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide. Nature. 363:159–163. http://dx.doi.org/10.1038/363159a0

Magrús-Chatnnet, A., H. Yu, S. Garcia, E. Duclos, B. Terris, and M. Bomsel. 2007. Galectosyl ceramide expressed on dendritic cells can mediate HIV-1 transfer from monocyte derived dendritic cells to autologous T cells. Virolology. 362:67–74. http://dx.doi.org/10.1016/j.virol.2006.11.035

Masterson, A.J., C.C. Sombock, T.D. De Grijui, Y.M. Graus, H.J. van der Vliet, S.M. Loughhead, A.J. van den Eertwegh, H.M. Pinedo, and R.J. Scheper. 2002. MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from CD34+ precursors. Blood. 100:701–703. http://dx.doi.org/10.1182/blood.V100.2.701

Mc Dermott, R., U. Ziylan, D. Spehner, H. Bausinger, D. Lipsker, M. Monnmaax, J.P. Cazenave, G. Raposo, B. Goud, H. de la Salle, et al. 2002. Birbeck granules are subdomains of endosomal recycling compartment in human epidermal Langerhans cells, which form where Langerin accumulates. Mol. Biol. Cell. 13:317–335. http://dx.doi.org/10.1091/mbc.01-06-0300

McCarthy, B.G., S.T. Hsieh, A. Stocks, P. Hauer, C. Macko, D.R. Cornblath, J.W. Griffin, and J.C. McArthur. 1995. Cutaneous innervation in sensory neuropathies: evaluation by skin biopsy. Neurology. 45:1848–1855. http://dx.doi.org/10.1212/wnl.45.10.1848

Moreno, M.J., J.A. Terron, D.B. Stambrooke, H. Doods, and E. Hamel. 2002. Characterization of calcitonin gene-related peptide (CGRP) receptors and their receptor-activity-modifying proteins (RAMPs) in human brain microvascular and astroglial cells in culture. Neuropharmacology. 42:270–280. http://dx.doi.org/10.1016/S0028-3908(01)00176-9
Olesen, J., H.C. Diener, I.W. Hustedt, P.J. Goadby, D. Hall, U. Meier, S. Pollentier, and L.M. Lesko; BIBN 4096 BS Clinical Proof of Concept Study Group. 2004. Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migrane. N. Engl. J. Med. 350:1104–1110. http://dx.doi.org/10.1056/NEJMoa030505

Poyner, D.R., P.M. Sexton, I. Marshall, D.M. Smith, R. Quirion, W. Born, R. Muff, J.A. Fischer, and S.M. Foord. 2002. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol. Rev. 54:233–246. http://dx.doi.org/10.1124/pr.54.2.233

Rosenfeld, M.G., J.J. Mermod, S.G. Amara, L.W. Swanson, P.E. Sawchenko, J. Rivier, W.W. Vale, and R.M. Evans. 1983. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature. 304:129–135. http://dx.doi.org/10.1038/304129a0

Sams, A., and I. Jansen-Olesen. 1998. Expression of calcitonin receptor-like receptor-activity-modifying proteins in human cranial arteries. Neurosci. Lett. 258:41–44. http://dx.doi.org/10.1016/S0304-3940(98)00844-1

Zhou, L., D.W. Kitch, S.R. Evans, P. Hauer, S. Raman, G.J. Ebenezer, M. Gerschenson, C.M. Marra, V. Valcour, R. Díaz-Arrastia, et al; NARC and ACTG A5117 Study Group. 2007. Correlates of epidermal nerve fiber densities in HIV-associated distal sensory polyneuropathy. Neurology. 68:2113–2119. http://dx.doi.org/10.1212/01.wnl.0000264888.87918.a1

Zhou, Z., N. Barry de Longchamps, A. Schnitt, M. Zerbib, M.C. Vacher-Lavenu, M. Bomsel, and Y. Ganor. 2011. HIV-1 efficient entry in inner foreskin is mediated by elevated CCL5/RANTES that recruits T cells and fuels conjugate formation with Langerhans cells. PLoS Pathog. 7:e1002100. http://dx.doi.org/10.1371 journal.ppat.1002100

Torii, H., J. Hosoi, S. Beisert, S. Xu, F.E. Fox, A. Asahina, A. Takashima, A.H. Rook, and R.D. Granstein. 1997. Regulation of cytokine expression in macrophages and the Langerhans cell-like line X532 by calcitonin gene-related peptide. J. Leukoc. Biol. 61:216–223.

Walker, C.S., A.C. Conner, D.R. Poyner, and D.L. Hay. 2010. Regulation of signal transduction by calcitonin gene-related peptide receptors. Trends Pharmacol. Sci. 31:476–483. http://dx.doi.org/10.1016/j.tips.2010.06.006

Schifter, S. 1997. Expression of the calcitonin gene family in medullary thyroid carcinoma. Peptides. 18:307–317. http://dx.doi.org/10.1016/S0196-9781(96)00169-6