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CGRP inhibits human Langerhans cells infection with HSV by differentially modulating specific HSV-1 and HSV-2 entry mechanisms

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Herpes simplex virus (HSV) is widespread globally, with both HSV-1 and HSV-2 responsible for genital herpes. During sexual transmission, HSV targets epithelial cells, sensory peripheral pain neurons secreting the mucosal neuropeptide calcitonin gene-related peptide (CGRP), and mucosal immune cells including Langerhans cells (LCs). We previously described a neuro-immune crosstalk, whereby CGRP inhibits LCs-mediated human immunodeficiency virus type 1 (HIV-1) transmission. Herein, to further explore CGRP-mediated anti-viral function, we investigated whether CGRP affects LCs infection with HSV. We found that both HSV-1 and HSV-2 primary isolates productively infect monocyte-derived LCs (MDLCs) and inner foreskin LCs. Moreover, CGRP significantly inhibits infection with both HSV subtypes of MDLCs and langerin^{high}, but not langerin^{low}, inner foreskin LCs. For HSV-1, infection is mediated via the HSV-1-specific entry receptor 3-O sulfated heparan sulfate (3-OS HS) in a pH-depended manner, and CGRP down-regulates 3-OS HS surface expression, as well as abrogates pH dependency. For HSV-2, infection involves langerin-mediated endocytosis in a pH-independent manner, and CGRP up-regulates surface expression of atypical langerin double-trimer oligomers. Our results show that CGRP inhibits mucosal HSV infection by differentially modulating subtype-specific entry receptors and mechanisms in human LCs. CGRP could turn out useful for prevention of LCs-mediated HSV infection and HSV/HIV-1 co-infection.

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INTRODUCTION

According to the World Health Organization, in 2016 around 3.7 and 0.5 billion people under the age of 50 (i.e. 67% and 13% of the world's population) were infected with herpes simplex virus (HSV)-1 and HSV-2, respectively, making HSV a highly prevalent sexually transmitted infection (STI)[1]. HSV-1 induces painful orofacial herpes, as well as life threatening meningitis, keratitis, encephalitis and neonatal infection[2]. Both HSV-2 and HSV-1 induce genital ulcers[3,4], and HSV-2 is the largest STI contributor and risk factor for increased HIV-1 acquisition during co-infection[5].

HSV sexual transmission occurs once the virus invades the protective mucosal barriers of genital epithelia and targets epithelial cells (i.e. epitheliotropic) in a lytic cycle[6]. Subsequently, HSV enters the nerve endings of sensory peripheral neurons (i.e. neurotropic), in which it establishes life-long latency. Episodes of recurrence occur when latent HSV is reactivated and transported back by anterograde axonal transport close to the initial site of infection[6]. HSV-1 establishes latency in a subset of sensory peripheral pain neurons termed nociceptors that secret the mucosal vasodilator neuropeptide calcitonin gene-related peptide (CGRP)[7]. Such latency is established both in human[8] and murine[9] CGRP^{+} nociceptors, in which latent HSV-1 decreases CGRP secretion[10]. In contrast, HSV-2 establishes latency in a different population of CGRP^{low} murine nociceptors[11].

Different types of resident immune cells within genital epithelia are also HSV and HIV-1 cellular targets, including antigen-presenting Langerhans cells (LCs). Indeed, LCs can be productively infected with HSV, leading to their apoptosis and transfer of HSV antigens to dermal dendritic cells (DCs), facilitating viral relay (reviewed in[12]). Previous studies (included our own) also demonstrated infection of LCs with HIV-1, e.g., at high HIV-1 doses or following cell activation[13–18], followed by transfer of intact infectious HIV-1 virions from LCs to CD4^{+} T-cells in a temporal manner[19,20]. During co-infection, HSV-2 competes with HIV-1 for binding to the LC-specific C-type lectin langerin, thereby decreasing langerin availability and langerin-mediated HIV-1 degradation[21]. HSV-2 also increases expression of the HIV-1 receptor CD4 and co-receptor CCR5 in LCs, thereby enhancing their susceptibility to HIV-1 infection[22,23].

Recent studies provide evidence for the complexity of the LC network within the epithelial compartment, by describing several cell subsets that can be distinguished by their expression levels of CD1a and langerin. These studies identified CD1a^{high}langerin^{high} LCs, termed LC1 by one study, in the epidermis of the inner

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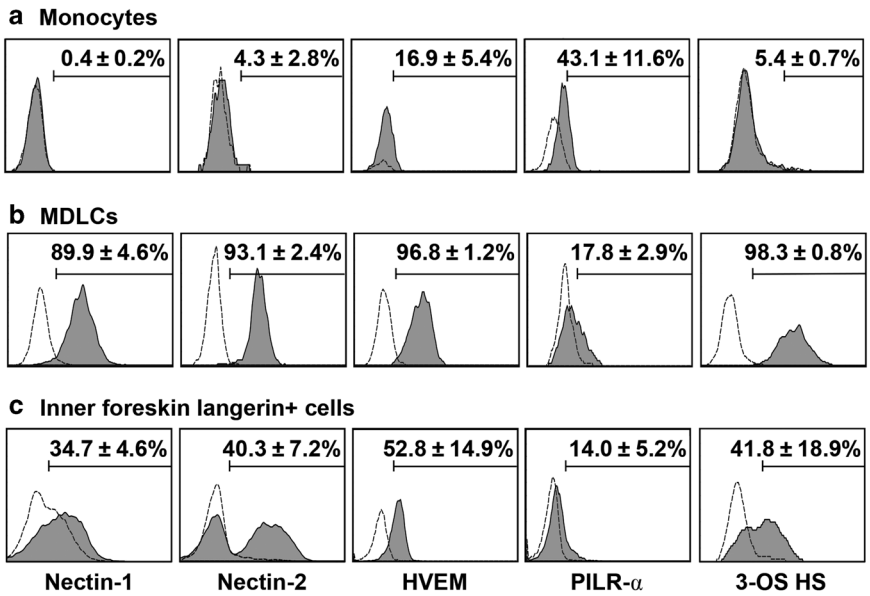

Fig. 1 Human LCs express HSV-1 and HSV-2 entry receptors. Monocytes (**a**), MDLCs (**b**), or inner foreskin epidermal cell suspensions (**c**), were labeled for cell-surface expression of HSV-1/2 entry receptors and langerin. Matched isotype control Abs (broken lines) served as negative controls, and cells were examined by flow cytometry. Shown are representative flow cytometry overlays, with numbers representing mean ± SEM ($n = 4$–9, see Supplementary Fig. 2) percentages of positive cells out of total cells (**a**) or gated on langerin$^+$ cells (**b**, **c**).

foreskin and skin[24,25]. Additional CD1a^{low}langerin$^{neg/low}$ cells were also identified, and combined with differential expression of CD11c or CD1c, were termed epidermal dendritic cells (Epi-cDC2s)[24] or LC2[25], respectively. As a proportion of Epi-cDC2s expresses langerin in the inner foreskin (but only a very low proportion expresses langerin in skin), coinciding with the high frequency of the LC2 subset in inner foreskin, it remains to be determined whether langerin-expressing Epi-cDC2s and LC2 represent the same or distinct LC subsets.

Sensing of and protecting from invading pathogens is orchestrated by a local mucosal neuro-immune crosstalk between immune cells and nociceptors, mediated via direct contacts and nociceptor-secreted neuropeptides, including CGRP[26]. This 37 amino acid neuropeptide is a potent vasodilator that plays important physiological and pathophysiological roles[7], and can directly modulate immune function in a vasodilator-independent manner. For instance, nociceptors associate with LCs and CGRP shifts LCs-mediated antigen presentation and cytokine secretion from Th1 to Th2/Th17[27]. We previously discovered that CGRP also exerts anti-viral activity, as it strongly inhibits LCs-mediated HIV-1 transfer to CD4$^+$ T-cells via a multitude of co-operative mechanisms[19,28,29]. Accordingly, CGRP increases langerin surface expression and facilitates efficient viral degradation, by diverting HIV-1 from endo-lysosomes towards faster viral proteasomal degradation. CGRP also decreases LCs expression of adhesion molecules, leading to reduced formation of conjugates with CD4$^+$ T-cells, to limit viral transfer.

Herein, to further explore the extent of CGRP-mediated anti-viral function, we investigated whether CGRP could affect infection of LCs with HSV. Our results show that CGRP significantly inhibits human LCs infection with both HSV-1 and HSV-2, via distinct effects that are related to modification of subtype-specific HSV entry receptors and mechanisms.

RESULTS

Human LCs express HSV-1 and HSV-2 entry receptors

HSV uses several cell surface receptors to mediate its cellular entry (reviewed in[30]). Some of these receptors are subtype-specific, namely paired-immunoglobulin-like receptor alpha (PILR-α) is specific to HSV-1, while nectin-2 is specific to HSV-2. In contrast, nectin-1 and herpes-virus entry mediator (HVEM) are not subtype-specific and can be used by both HSV-1 and HSV-2. In addition, heparan sulfate (HS) proteoglycans are attachment receptors for both HSV-1 and HSV-2, yet the 3-O sulfated form of HS (3-OS HS) serves as an HSV-1-specific fusion receptor.

To test for expression of HSV receptors, human monocytes (i.e., LC precursors under inflammatory conditions) and monocyte-derived LCs (MDLCs) were surface labeled using specific antibodies (Abs) directed against the abovementioned HSV entry receptors and langerin, and analyzed by flow cytometry. As shown in Fig. 1, monocytes expressed negligible levels of nectin-1, nectin-2 and 3-OS HS, while approximately 15% expressed HVEM and 45% expressed PILR-α (Fig. 1a). Differentiation of monocytes into MDLCs resulted, as expected, in high CD1a and loss of CD14 expression in >95% of MDLCs, as well as expression of langerin in approximately 30% of MDLCs (Supplementary Figure 1a). Monocytes-to-MDLCs differentiation also reduced PILR-α, but increased HVEM and induced nectin-1, nectin-2 and 3-OS HS expression on ≥90% MDLCs (Fig. 1b and Supplementary Figure 2).

We next confirmed the presence of CD1a^{high}langerin^{high} and CD1a^{low}langerin^{low} LC subsets within langerin$^+$ inner foreskin epidermal cells, we termed herein langerin^{high} or langerin^{low} cells (Supplementary Fig. 1b). Of note, based on their expression of high and uniform CD1a levels, MDLCs cannot be separated into similar subsets and were therefore gated, below and in ensuing experiments, on all langerin$^+$ MDLCs (Supplementary Fig. 1a). Compared to MDLCs, inner foreskin langerin$^+$ cells (i.e. including langerin^{high} and langerin^{low} cells together) expressed all HSV entry receptors tested, but at lower levels (Fig. 1c). While langerin^{high} and langerin^{low} cells expressed similar levels of HVEM, PILR-α and 3-OS HS, langerin^{high} cells expressed higher nectin-1 and lower nectin-2 (Supplementary Fig. 2).

These results indicate that human LCs might be permissive to infection with both HSV-1 and HSV-2.

Human LCs are productively infected with both HSV-1 and HSV-2

To evaluate HSV infection of human LCs, we pulsed MDLCs for 2 h with primary isolates of either HSV-1 or HSV-2. The cells were next

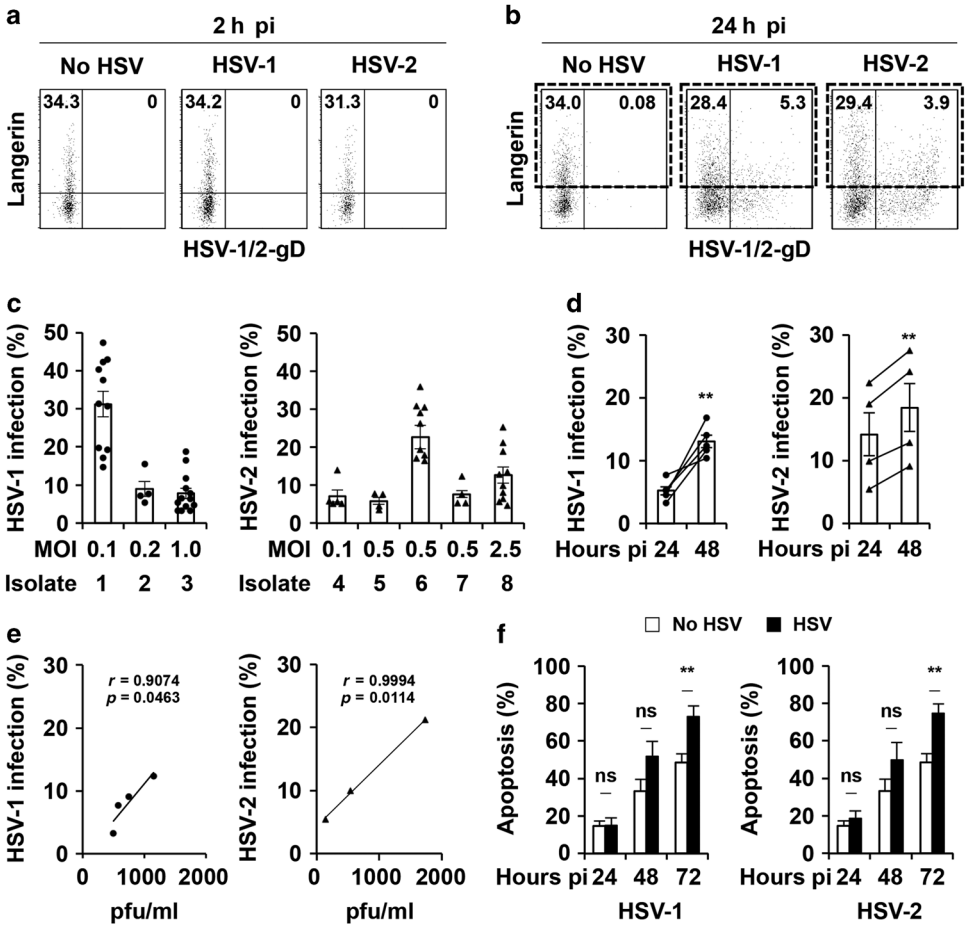

Fig. 2 Human MDLCs are productively infected with HSV-1 and HSV-2. a–d MDLCs were pulsed for 2 h with different primary isolates of HSV-1 or HSV-2, and infection was examined 24–48 h pi by flow cytometry. In (**a**, **b**), shown are representative flow cytometry dot-plots of MDLCs infected with HSV-1 or HSV-2 vs. no HSV control, and examined immediately following the 2 h viral pulse (**a**) or 24 h pi (**b**). Numbers represent percentages of langerin$^+$HSV-1/2-gD$^-$ (upper left regions) and langerin$^+$HSV-1/2-gD$^+$ (upper right regions) cells. In (**c**, **d**), shown are mean ± SEM percentages of infection, calculated as ([langerin$^+$HSV-1/2-gD$^+$ MDLCs]/[total langerin$^+$ MDLCs] x 100), for each isolate tested at the indicated MOIs and examined at 24 h pi (**c**), and for MDLCs pulsed with HSV-1 (isolate 3 at MOI = 1.0) or HSV-2 (isolates 6/7 at MOI = 0.5) and examined 24–48 h pi (**d**). Dots (for HSV-1) or triangles (for HSV-2) represent independent experiments, using MDLCs from different individuals. **e** MDLCs were pulsed for 2 h with HSV-1 (isolate 2 at MOI = 0.2 and isolate 3 at MOI = 1.0) or HSV-2 (isolates 6/7 at MOI = 0.5). At 24 h pi, infection was examined by flow cytometry and replication was determined by plaque assay of the culture supernatants. Lines denote linear correlations between HSV-1/2 infection percentages and plaque-forming units (pfu)/ml released into the culture supernatants, with r and p values derived from Pearson correlations. **f** MDLCs were pulsed for 2 h with HSV-1 (isolate 3 at MOI = 1.0) or HSV-2 (isolates 6/7 at MOI = 0.5) and apoptosis was examined 24–72 h pi by flow cytometry. Shown are mean ± SEM (derived from $n = 7$ independent experiments using MDLCs from different donors) percentages of apoptosis, calculated as ([langerin$^+$Annexin$^+$PI$^-$ MDLCs]/[total langerin$^+$ MDLCs] x 100). In all graphs, $^{**}p < 0.0050$; two-tailed Student's t-test.

washed with low pH buffer in order to remove surface-bound virions, stained for surface langerin and intracellular HSV-1/2-gD (i.e., an HSV-1/2 envelope glycoprotein that is a late gene product, whose expression is indicative of productive infection), and examined by flow cytometry.

These experiments showed that while MDLCs were not infected immediately following the 2 h viral pulse period (Fig. 2a), a double langerin$^+$HSV-1/2-gD$^+$ population could be observed 24 h post-infection (pi) with both HSV subtypes (Fig. 2b). As expected, using primary isolates of HSV, infection was isolate-dependent, i.e. different primary isolates used at either low or high multiplicities of infection (MOIs) induced varying degrees of infection (Fig. 2c), calculated as the percentage of langerin$^+$HSV-1/2-gD$^+$ cells out of total langerin$^+$ MDLCs (broken line frames in Fig. 2b). In addition, both HSV-1 and HSV-2 infection of MDLCs was time-dependent (Fig. 2d).

To demonstrate that MDLCs infection was productive, we collected their culture supernatants and measured the amounts of secreted infectious virus by standard plaque assay using HSV-permissive Vero cells. These experiments showed that MDLCs released infectious HSV-1 and HSV-2 at 24 h pi, at levels that correlated with that measured by HSV-1/2-gD intracellular staining (Fig. 2e).

Finally, we evaluated HSV-induced apoptosis of human LCs by flow cytometry. These experiments showed that the proportion of apoptotic MDLCs (i.e., defined as langerin$^+$ Annexin$^+$ Propidium Iodide (PI)$^-$ cells) increased over time following HSV infection, and was significantly higher for both HSV subtypes only at 72 h pi, compared to non-infected MDLCs (Fig. 2f). At these different time points, cell viability of both non-infected and HSV-infected langerin$^+$ MDLCs was of approximately 90% (Supplementary Figure 3). Therefore, in subsequent experiments using MDLCs, HSV infection was measured at 24–48 h pi to ensure the absence of notable apoptosis.

To further extend the findings obtained with MDLCs, we pulsed inner foreskin epidermal cell suspensions for 24 h with primary

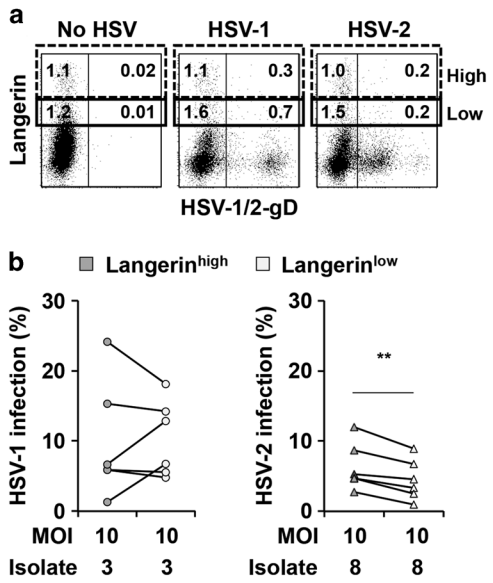

Fig. 3 Human inner foreskin langerin-expressing cells are productively infected with HSV-1 and HSV-2. Inner foreskin epidermal cell suspensions were pulsed for 24 h with primary isolates of HSV-1 (isolate 3) or HSV-2 (isolate 8) at MOI = 10.0, stained at 48 h pi for surface langerin and intracellular HSV-1/2-gD, and examined by flow cytometry. **a** Representative flow cytometry dot-plots of suspensions infected with HSV-1 or HSV-2 vs. no HSV control, with numbers representing percentages of langerin^{high}HSV-1/2^{-} (upper left regions), langerin^{high}HSV-1/2^{+} (upper right regions), langerin^{low}HSV-1/2^{-} (middle left regions) and langerin^{low}HSV-1/2^{+} (middle right regions) cells. **b** Matched infection percentages of inner foreskin langerin^{high} (dark grey) vs. langerin^{low} (light grey) cells, infected with either HSV-1 (dots) or HSV-2 (triangles). For HSV-2, $^{**}p = 0.0023$; paired two-tailed Student's t-test.

isolates of either HSV-1 or HSV-2, and evaluated infection at 48 h pi by flow cytometry as above. These experiments showed that langerin^{high} and langerin^{low} cells (see the gating strategy in Supplementary Fig. 1b) could be infected with both HSV-1 and HSV-2 (Fig. 3a). We next calculated infection separately for langerin^{high} and langerin^{low} cells, as the percentages of langerin^{high}HSV-1/2-gD^{+} out of total langerin^{high} (broken line frames in Fig. 3a) or langerin^{low}HSV-1/2-gD^{+} out of total langerin^{low} cells (solid line frames in Fig. 3a). This analysis showed that HSV-1 infection of langerin^{high} cells did not significantly differ from that of langerin^{low} cells (Fig. 3b), with mean ± SEM (derived from $n = 6$ independent experiments using different inner foreskin tissues) infection percentages of 9.9 ± 3.4 vs. 10.4 ± 2.2. In contrast, HSV-2 infection of langerin^{high} cells was significantly higher than that of langerin^{low} cells (6.3 ± 1.4 vs. 4.5 ± 1.2, $p = 0.0023$, paired Student's t-test), suggestive of langerin usage by HSV-2.

These results demonstrate that human LCs are productively infected with both HSV-1 and HSV-2.

CGRP inhibits human LCs infection with both HSV-1 and HSV-2

To investigate the potential impact of CGRP on HSV infection, MDLCs were left untreated or pre-treated for 24 h with different molar concentration of CGRP, within its effective concentration range that significantly inhibits MDLCs-mediated HIV-1 transfer to CD4^{+} T-cells, as we previously showed[29]. MDLCs were also pre-treated with SAX, a metabolically stable analogue of CGRP, which inhibits MDLCs-mediated HIV-1 transfer, as we recently reported[31]. The cells were then pulsed with primary isolates of HSV-1 or HSV-2 for 2 h and infection was examined 24–48 h pi by flow cytometry as above. To account for variations related to the use of MDLCs from different donors that were infected with different HSV-1/2 primary isolates at different MOIs (as shown in Fig. 2c), infection was normalized to that of untreated cells serving as the 100% set point and averaged.

These experiments showed that CGRP and SAX dose-dependently inhibited MDLCs infection at 24 h pi, with both HSV-1 and HSV-2 (Fig. 4a, b; see Supplementary Fig. 4 for the separate non-normalized dose-responses curves). For both HSV subtypes, SAX-mediated inhibition was more potent than that of CGRP, with respective pIC_{50} potencies [95% confidence intervals] of 9.5 [10.5-8.8] vs. 7.5 [9.2-5.1] for HSV-1, and 7.1 [8.7-5.4] vs. 5.8 [7.2-2.3] for HSV-2. In addition, CGRP-mediated inhibition was time-dependent, reaching 70–75% inhibition at 48 h pi for both HSV-1 and HSV-2 (Fig. 4c). Importantly, CGRP also significantly inhibited the release of infectious HSV-1 and HSV-2 at 24 h pi (Fig. 4d). Of note, as only a proportion of MDLCs expresses langerin (Supplementary Fig. 1) that is increased following CGRP pre-treatment (as we previously reported[29] and confirmed below), the inhibitory effect of CGRP on HSV-1 and HSV-2 infection of MDLCs might be attributed to increased percentages of langerin^{+} MDLCs. Yet, CGRP pre-treatment resulted in decreased percentages of langerin^{+}HSV-1/2-gD^{+} double-positive cells (data not shown), indicating actual inhibition of MDLCs infection.

We next used inner foreskin epidermal cell suspensions, which were left untreated or pre-treated for 24 h with CGRP. The cells were then pulsed with primary isolates of HSV-1 or HSV-2 for 24 h, and infection was examined 48 h pi by flow cytometry. As for MDLCs, infection was normalized to that of untreated cells, in order to account for variations related to the use of tissues from different donors (as shown in Fig. 3b). These experiments showed that following CGRP pre-treatment, infection was significantly decreased for both HSV subtypes in langerin^{high} cells (Fig. 4e). In contrast, CGRP had no effect on infection of langerin^{low} cells with either HSV-1 or HSV-2 (Fig. 4f).

These results demonstrate that CGRP exerts anti-viral activities during human LCs infection with both HSV-1 and HSV-2, and inhibits infection of MDLCs and langerin^{high}, but not langerin^{low}, inner foreskin LCs.

CGRP inhibits 3-OS HS-dependent and pH-dependent HSV-1 infection of human LCs

We next used MDLCs in order to characterize HSV-1 receptor usage, entry mechanism and CGRP-mediated inhibition. To determine which receptors mediate HSV-1 infection, we first pre-incubated HSV-permissive Vero cells for 1 h at 37 °C with neutralizing Abs (nAbs) to HSV-1 entry receptors (i.e., nectin-1, HVEM and PILR-α) and confirmed by plaque assay their capacity to inhibit infection with HSV-1 primary isolates (Supplementary Fig. 5). We then pre-incubated untreated and CGRP-treated MDLCs with the HSV-1 entry receptor nAbs, as well as with a langerin nAb (i.e., as one recent study reported langerin usage by HSV-1 in abdominal skin LCs[32]). MDLCs were also pre-incubated with heparinase, which cleaves HS / 3-OS HS[33], as we confirmed herein (Supplementary Fig. 6). The cells were next pulsed for 2 h with HSV-1 primary isolates and infection was measured 24 h pi by flow cytometry. These experiments showed that none of the nAbs could significantly block HSV-1 infection of untreated MDLCs (Fig. 5a). In contrast, pre-treatment with heparinase strongly inhibited HSV-1 infection (Fig. 5a). Importantly, heparinase decreased by almost 80% not only the set-up infection of untreated cells, but also the reduced infection of CGRP-treated cells (Fig. 5b). We next tested the effect of CGRP on 3-OS HS expression and found that CGRP significantly decreased the mean fluorescent intensity (MFI) of 3-OS HS surface expression in MDLCs (Fig. 5c). In contrast, CGRP treatment had no effect on expression of other HSV-1/2 entry receptors (Supplementary Fig. 7).

To further extend these observations, we pre-incubated untreated and CGRP-treated inner foreskin epidermal cell suspensions with heparinase or the langerin nAb, followed by 24 h pulse with primary isolates of HSV-1 and evaluation of

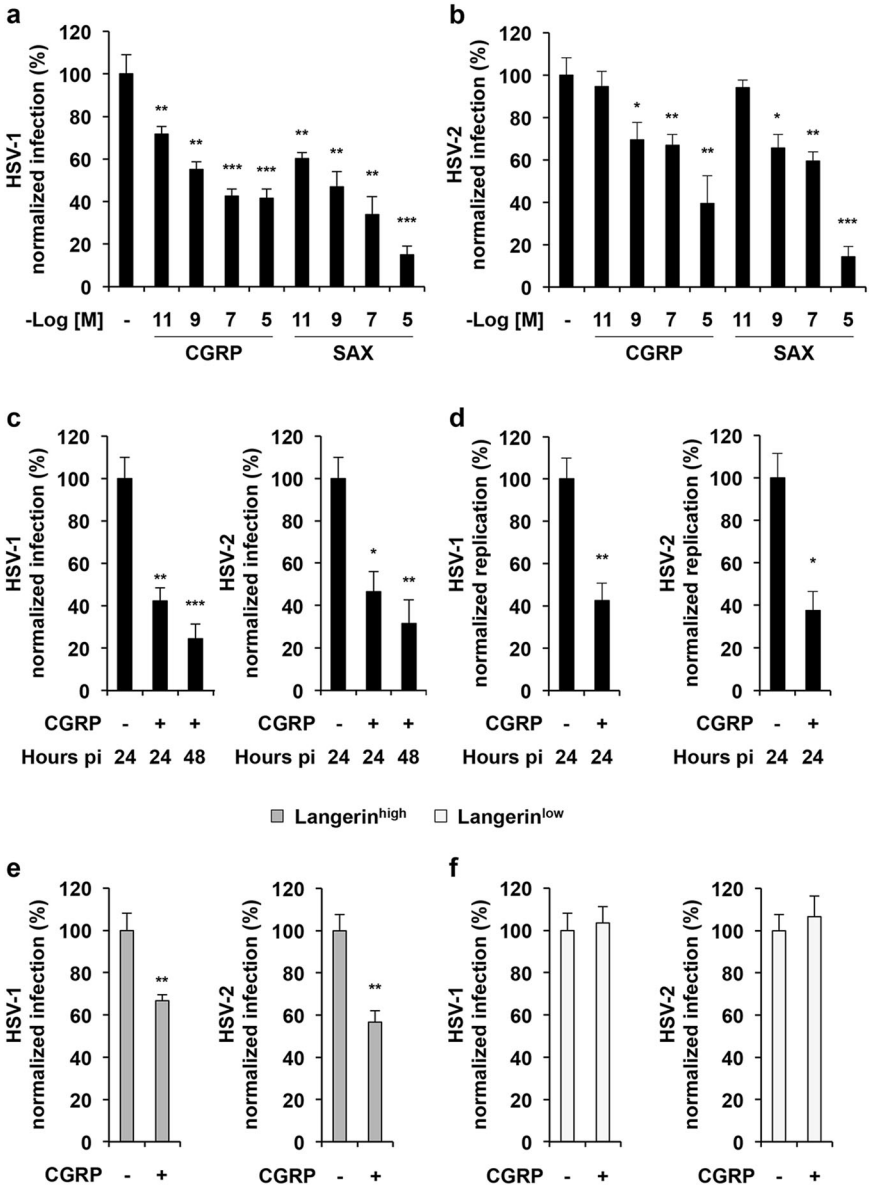

Fig. 4 CGRP inhibits human LCs infection with HSV-1 and HSV-2. a–d MDLCs were left untreated or pre-treated for 24 h with CGRP and SAX at the indicated molar concentrations (**a**, **b**), or with 100 nM CGRP (**c**, **d**). Cells were then pulsed for 2 h with primary isolates of HSV-1 (isolate 2 at MOI = 0.2 and isolate 3 at MOI = 1.0) or HSV-2 (isolates 6/7 at MOI = 0.5), and further incubated for 24 h (**a**, **b**, **d**) or 24–48 h (**c**). HSV-1/2 infection was examined by flow cytometry and replication was determined by plaque assay of the culture supernatants. Results represent mean ± SEM ($n = 3$–4) percentages of infection (**a**–**c**) or replication (**d**), normalized against untreated cells serving as the 100% set point. **e**, **f** Inner foreskin epidermal cell suspensions were left untreated or pre-treated for 24 h with 1 μM CGRP, pulsed for 24 h with HSV-1 (isolate 3) or HSV-2 (isolate 8) at MOI = 10.0, and infection was examined 48 h pi by flow cytometry. Results represent mean ± SEM (derived from $n = 6$ independent experiments using tissues from different donors) normalized percentages of infection of langerin^{high} (**e**) and langerin^{low} (**f**) cells. In all graphs, $^{*}p < 0.0500$, $^{**}p < 0.0050$ and $^{***}p < 0.0005$ vs. untreated; two-tailed Student's *t*-test.

infection at 48 h pi by flow cytometry. Focusing on langerin^{high} cells, in which CGRP inhibits HSV-1 infection (see Fig. 4e), these experiments showed that heparinase significantly decreased HSV-1 infection in both untreated and CGRP-treated langerin^{high} cells (Fig. 5d). In contrast to MDLCs, the langerin nAb could inhibit HSV-1 infection in langerin^{high} cells (Fig. 5d). CGRP also decreased 3-OS HS surface expression in langerin^{high} cells, with mean ± SEM percentages of 3-OS HS-positive cells of 10.2 ± 1.5 vs. 6.2 ± 0.8 (as evaluated in epidermal suspensions of two individuals).

These results show that HSV-1 uses 3-OS HS to enter untreated and CGRP-treated MDLCs, and both 3-OS HS and langerin in inner foreskin langerin^{high} LCs.

Following receptor binding, HSV-1/2 entry is mediated by pH-independent fusion with the plasma membrane, and/or endocytic fusion that can be pH-independent or low pH-dependent[34]. We therefore pulsed untreated and CGRP-treated MDLCs with HSV-1 primary isolates, in the absence or presence of inhibitors of endolysosomal acidification, namely the lysosomotropic agent NH_4Cl and the inhibitor of ATPase H^+ pumps bafilomycin A1, and evaluated infection 24 h pi by flow cytometry. These experiments showed that in untreated MDLCs, the inhibitors had no effect on HSV-1 infection when included only during the 2 h viral pulse period (Fig. 5e), but significantly inhibited HSV-1 infection when included during the 24 h CGRP treatment period (Fig. 5f). In contrast,

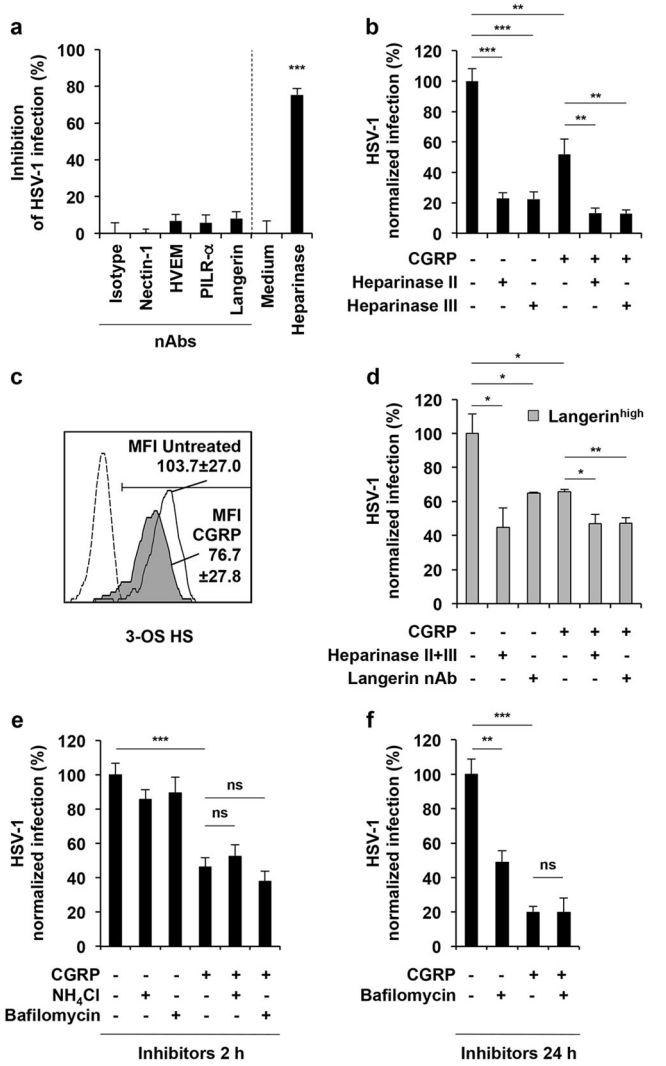

Fig. 5 CGRP decreases 3-OS HS expression and abrogates pH dependency during HSV-1 infection of human LCs. a, **b** Untreated or CGRP-treated MDLCs were pre-incubated for 1 h at 37 °C with nAbs to nectin-1, HVEM, PILR-α or langerin vs. matched isotype non-neutralizing control Abs, or pre-incubated with heparinase II/III vs. medium. Cells were then pulsed for 2 h with HSV-1 (isolate 3 at MOI = 1.0) and infection was examined 24 h pi by flow cytometry. Results represent mean ± SEM percentages of HSV-1 infection inhibition (**a**, $n = 7$) or normalized against untreated cells (**b**, $n = 5$). **c** Representative flow cytometry overlay, showing surface expression of 3-OS HS in untreated (empty histogram) and CGRP-treated (filled grey histogram) MDLCs vs. isotype control (broken line). Numbers represent mean ± SEM ($n = 3$) MFIs of 3-OS HS expression, gated on langerin$^+$ MDLCs. **d** Untreated or CGRP-treated inner foreskin epidermal cell suspensions were pre-incubated for 1 h at 37 °C with heparinase II + III or langerin nAb. Cells were then pulsed for 24 h with HSV-1 (isolate 3 at MOI = 10.0), and infection was examined 48 h pi by flow cytometry. Results represent mean ± SEM (using $n = 3$ tissues from different individuals) percentages of HSV-1 infection normalized against untreated cells. **e**, **f** Untreated or CGRP-treated MDLCs were pulsed with HSV-1 (isolates 1 at MOI = 0.1 and isolate 2 at MOI = 0.2), alone or in the presence of NH_4Cl or bafilomycin A1 during the 2 h pulse period (**e**) or 24 h CGRP treatment period (**f**), and infection was measured 24 h pi by flow cytometry. Results represent mean ± SEM ($n = 3$–5) percentages of HSV-1 infection normalized against untreated cells. In all graphs, $*p < 0.0500$, $**p < 0.0050$ and $***p < 0.0005$; two-tailed Student's *t*-test.

the inhibitors had no effect in CGRP-treated MDLCs, when added either during the pulse or CGRP treatment periods (Fig. 5e, f).

Together, these results show that HSV-1 enters human LCs in 3-OS-dependent and pH-dependent manners. GCRP inhibits HSV-1 infection in LCs by potentially decreasing 3-OS HS surface expression and by abrogating the pH dependency required for infection.

CGRP inhibits langerin-dependent and pH-independent HSV-2 infection of human LCs

In addition to nectin-1, nectin-2 and HVEM that mediate HSV-2 entry, HSV-2 also binds and uses langerin as an entry receptor in LCs[21]. We therefore performed additional receptor blocking experiments as above, using nAbs to HSV-2 entry receptors and langerin. These experiments showed that pre-incubation with both nectin-1 and langerin nAbs significantly inhibited by approximately 15% and 50%, respectively, HSV-2 infection of untreated MDLCs, while the other nAbs had no significant effect (Fig. 6a). Of note, heparinase pre-treatment inhibited HSV-2 infection of untreated MDLCs by approximately 20% (Fig. 6a), which could be expected as HSV-2 uses HS, but not 3-OS HS, as an early attachment receptor[35]. Importantly, in CGRP-treated MDLCs, pre-incubation with the langerin nAb also reduced HSV-2 infection by approximately 50% (Fig. 6b). Similarly, pre-incubation with the langerin nAb significantly inhibited HSV-2 infection of both untreated and CGRP-treated inner foreskin langerin^{high} LCs (Fig. 6c). These results show that HSV-2 uses langerin to enter untreated and CGRP-treated MDLCs and inner foreskin langerin^{high} LCs.

We next investigated whether CGRP affects clathrin/caveolin-mediated endocytosis that is involved in langerin internalization and recycling following ligand binding[36,37], and determined the pH-dependency of HSV-2 entry. Accordingly, untreated and CGRP-treated MDLCs were pulsed with HSV-2 primary isolates for 2 h, in the absence or presence of dynasore (a dynamin inhibitor that blocks clathrin-mediated endocytosis), methyl beta cyclodextrin (MβCD, a cholesterol depleting agent that blocks caveolin-mediated endocytosis) or NH_4Cl and bafilomycin A1 as above. These experiments showed that dynasore or MβCD reduced HSV-2 infection, in both untreated and CGRP-treated MDLCs (Fig. 6d and Supplementary Figure 8). In contrast, NH_4Cl and bafilomycin A1 had no effect on HSV-2 infection in either untreated or CGRP-treated MDLCs (Fig. 6e, f). These results indicate that HSV-2 enters MDLCs via langerin-dependent pH-independent endocytosis.

As we previously reported, CGRP increases langerin expression in MDLCs[29]. To confirm these observations and understand why langerin up-regulation appears to decrease rather than increase HSV-2 entry in LCs, we investigated whether CGRP affects langerin trimeric structure[38]. Hence, we cross-linked surface langerin in MDLCs using non-saturating concentration of the chemical cross-linker disuccinimidyl glutamarate (DSG), and evaluated langerin oligomeric status by Western blot. These experiments showed that langerin was dissociated into its monomeric form in untreated MDLCs in the absence of cross-linking (Fig. 6g, lane 1). As expected, following cross-linking, langerin was present both as monomers and trimers, i.e., a band of approximately 120 kDa that equals three langerin monomers (Fig. 6g, lane 2). In line with our previous findings, CGRP significantly increased langerin expression in MDLCs (Fig. 6g, lane 3). Surprisingly, in addition to langerin monomers and trimers, a langerin higher molecular weight band corresponding to double trimers of approximately 240 kDa was detected in CGRP-treated and cross-linked MDLCs (Fig. 6g, lane 4). These double trimers were significantly increased, while monomers were decreased and trimers remained unchanged, when comparing CGRP-treated to untreated MDLCs following cross-linking (Fig. 6g, pie plots), suggesting *de-novo* formation of double trimers from monomers. Like CGRP, its metabolically stable analogue SAX also increased the expression of langerin double trimers in MDLCs (Supplementary Fig. 9).

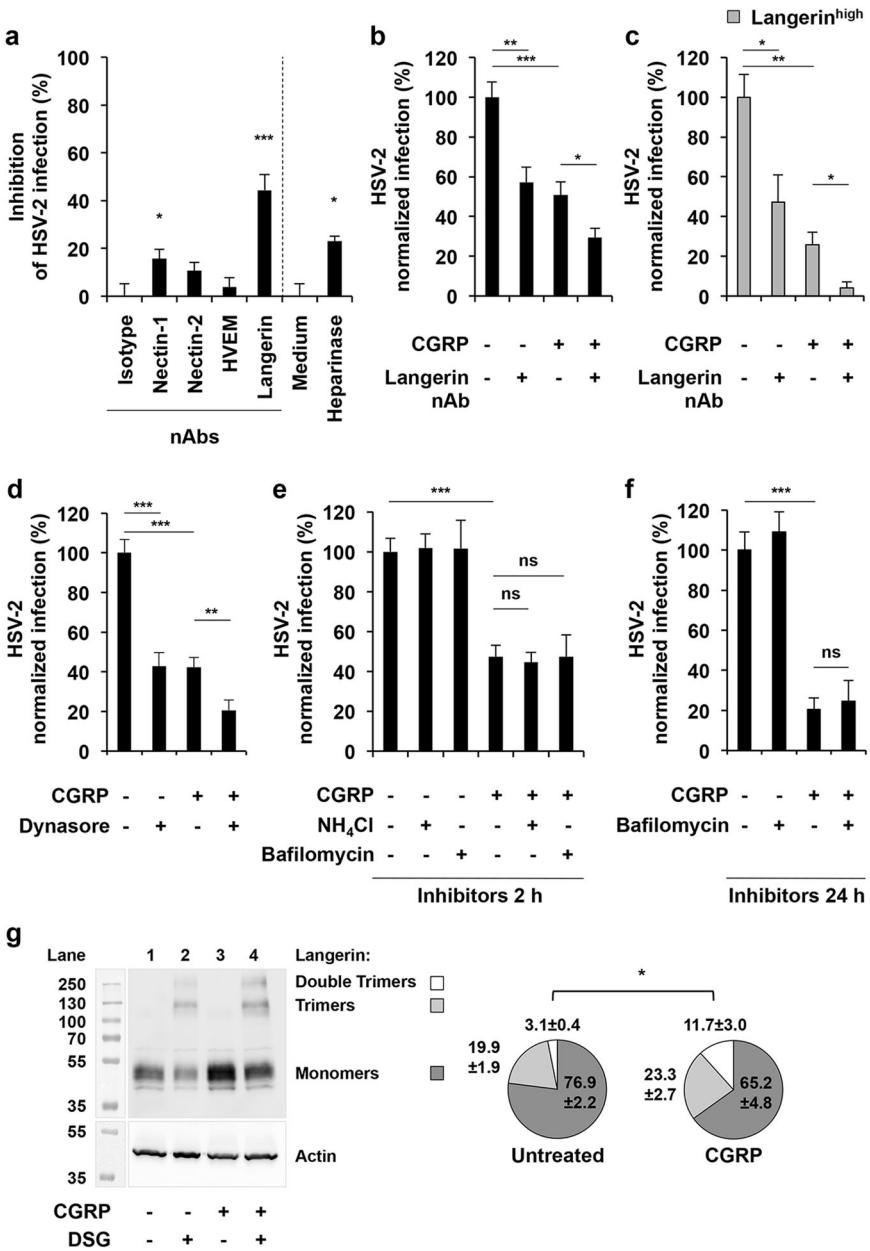

Fig. 6 CGRP inhibits langerin-mediated HSV-2 infection of human LCs and induces atypical langerin double trimers. a, **b** Untreated or CGRP-treated MDLCs were pre-incubated for 1 h at 37 °C with nAbs to nectin-1, nectin-2, HVEM or langerin vs. matched isotype non-neutralizing control Abs, or pre-incubated with heparinase II/III vs. medium. Cells were then pulsed for 2 h with HSV-2 (isolates 6 at MOI = 0.5 and isolate 8 at MOI = 2.5) and infection was examined 24 h pi by flow cytometry. Results represent mean ± SEM percentages of HSV-2 infection inhibition (**a**, $n = 8$) or normalized against untreated cells (**b**, $n = 6$). **c** Untreated or CGRP-treated inner foreskin epidermal cell suspensions were pre-incubated for 1 h at 37 °C with langerin nAb, pulsed for 24 h with HSV-2 (isolate 7 at MOI = 10.0), and infection was examined 48 h pi by flow cytometry. Results represent mean ± SEM (using $n = 3$ tissues from different individuals) normalized percentages of HSV-2 infection. **d**–**f** Untreated or CGRP-treated MDLCs were pulsed with HSV-2 (isolate 4 at MOI = 0.1 and isolate 7 at MOI = 0.5), alone or in the presence of dynasore, NH_4Cl or bafilomycin A1 as indicated, and infection was measured 24 h pi by flow cytometry. Results represent mean ± SEM ($n = 5$) normalized percentages of HSV-2 infection. In all graphs, *$p < 0.0500$, **$p < 0.0050$ and ***$p < 0.0005$; two-tailed Student's t-test. **g** Representative Western blot showing langerin expression in untreated (lane 1), untreated / DSG cross-linked (lane 2), CGRP-treated (lane 3), and CGRP-treated / DSG cross-linked (lane 4) MDLCs. Langerin appears as ~47 kDa monomers, ~120 kDa trimers and ~240 double trimers. Pie charts (representative of $n = 4$ experiments) show quantitative analysis of the proportions of each langerin form ($p = 0.0038$, ANOVA, untreated vs. CGRP-treated).

Together, these results show that HSV-2 enters MDLCs via langerin-mediated endocytosis and pH-independent mechanisms. CGRP might inhibit HSV-2 infection in MDLCs by inducing formation of atypical langerin trimers, which could prevent HSV-2 binding and subsequent entry.

DISCUSSION

In the present study, we identified a novel mechanism limiting mucosal HSV infection, involving neuroimmune interactions between CGRP and human LCs, as schematically summarized in Fig. 7. To the best of our knowledge, this is

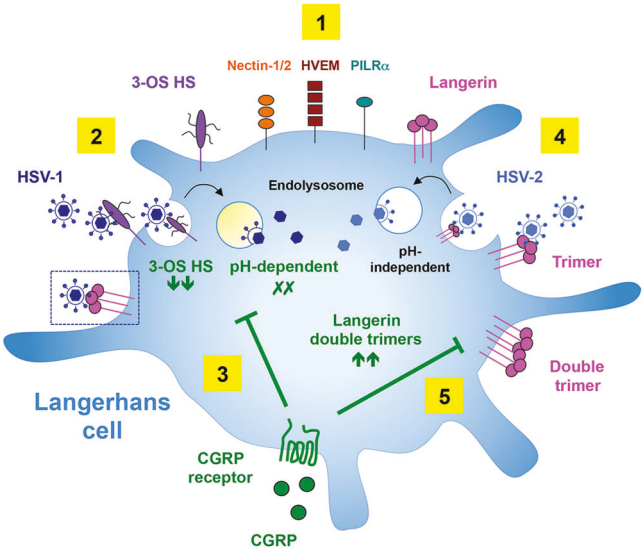

Fig. 7 Summary of HSV-1 and HSV-2 infection mechanisms in human LCs, and their inhibition by CGRP. (1) Human inner foreskin LCs and MDLCs express HSV-1/2 entry receptors and langerin; (2) HSV-1 uses 3-OS HS to enter MDLCs via a pH-dependent endocytic mechanism, as well as langerin in inner foreskin langerin^{high} LCs (broken line); (3) CGRP might inhibit HSV-1 infection by down-regulating 3-OS HS surface expression and abrogating pH dependency; (4) HSV-2 uses langerin to enter LCs, leading to clathrin/caveolin-mediated internalization and fusion in a pH-independent manner; (5) CGRP might inhibit HSV-2 infection by inducing formation of atypical high molecular weigh langerin double trimers.

the first report of a protective anti-viral role of CGRP during human HSV infection.

We show that human MDLCs are HSV-1/2-permissive and that CGRP significantly inhibits their productive infection with both HSV-1 and HSV-2. Such inhibition is of approximately 50% at 24 h pi, and reaches around 70–75% at 48 h pi for both viruses, indicating that the effect of CGRP is long lasting. Similarly, inhibition of MDLCs-mediated HSV-1 and HSV-2 infection at 24 h pi by SAX, a metabolically stable peptide analogue of CGRP that has a longer half-life[39,40], is more potent compared to CGRP. Hence, while SAX has lower potency than CGRP in inducing beneficial cardiovascular effects[40,41], and inhibiting LCs-mediated HIV-1 transfer to CD4^{+} T-cells as we recently reported[31], SAX might be preferable for potential clinical applications in the context of mucosal HSV infection.

In our experiments with human inner foreskin mucosal tissues, we could confirm the presence of two recently described LC subsets[24,25], namely CD1a^{high}langerin^{high} and CD1a^{low}langerin^{low} LCs. In comparison, all MDLCs are CD1a^{high} and a proportion of MDLCs expresses varying levels of langerin. Yet, langerin expression in MDLCs is a dynamic continuum that can be further increased depending on the in-vitro culture conditions, for instance in the absence of serum or following short exposure to certain cytokines[42,43]. We therefore speculate that MDLCs might be more representative of CD1a^{high}langerin^{high} tissue LCs, which also corresponds with our findings that CGRP inhibits infection of inner foreskin langerin^{high} cells with HSV-1 and HSV-2.

Inner foreskin langerin^{low} LCs might represent the proportion of Epi-cDC2s that expresses langerin and/or the LC2 subset, which are readily found in inner foreskin but at very low frequency in skin[24,25]. Our langerin-based gating strategy might explain why we observed similar HSV-1 infection degrees of langerin^{high} and langerin^{low} LCs in the inner foreskin, compared to the enhanced HSV-1 infection of langerin-negative Epi-cDC2s vs. langerin^{high} LCs recently reported in skin[32]. Interestingly, we found that CGRP has no effect on HSV-1 and HSV-2 infection of inner foreskin langerin^{low} cells. As Epi-cDC2s transcriptionally resemble DCs and not LCs[24,32], the lack of inhibitory effect of CGRP on langerin^{low} cells might correlate with our observations, showing that CGRP has no effect on monocyte-derived DCs (MDDCs)-mediated HIV-1 transfer to T-cells[29] and HSV-1/2 infection (data not shown). Further studies are now required in order to determine HSV-2 permissiveness of skin LCs vs. Epi-cDC2s, and investigate whether langerin^{low} vs. langerin^{neg} Epi-cDC2s in the inner foreskin represent distinct cell populations.

We further show that HSV-1 and HSV-2 rely on the usage of different entry receptors in MDLCs, namely the HSV-1-specific entry receptor 3-OS HS mediates HSV-1 infection, while langerin is the primary receptor mediating HSV-2 infection, highlighting its crucial role in conferring HSV-2 permissiveness of MDLCs. As we could not obtain complete blocking of HSV-1/2 infection in MDLCs when combining several nAbs simultaneously (data not shown), other surface receptors could mediate viral entry in MDLCs (e.g. the C-type lectin DC-SIGN[44], expressed by MDLCs but not tissue LCs, and potentially others that remain to be identified).

Although the yield of inner foreskin LCs is insufficient for full receptor neutralization experiments, we found higher HSV-2 infection and confirmed langerin usage by HSV-2 in langerin^{high} inner foreskin LCs. We could also show usage by HSV-1 in langerin^{high} inner foreskin LCs of both 3-OS HS and langerin, the latter recently described for skin LCs[32]. As langerin does not seem to contribute to HSV-1 infection in MDLCs, we speculate that such discrepancies could be explained in part by the fact that MDLCs are >95% positive for 3-OS HS and only partially express langerin, while tissue LCs are 100% positive for langerin and only partially express 3-OS HS (e.g., 25% of inner foreskin langerin^{high} cells as we show herein). Such different receptors abundance might affect langerin vs. 3-OS HS usage for HSV-1 entry.

Our results further indicate that the inhibitory effects of CGRP in MDLCs are exerted by distinct mechanisms, related to the specific HSV-1 and HSV-2 entry receptors we identified.

For HSV-1, we found that the virus uses 3-OS HS to enter MDLC, and in a pH-dependent manner alike described for skin LCs[32]. We hence speculate that HSV-1 entry takes place via binding to 3-OS HS at the plasma membrane, leading to 3-OS HS clustering within lipid rafts and its internalization[45], followed by low pH endocytic escape of HSV-1 into the cytosol. In turn, CGRP pre-treatment inhibits HSV-1 infection of MDLCs by potentially affecting two different steps in this entry process, namely CGRP significantly decreases 3-OS HS expression and also abrogates pH dependency. Although the exact mechanism through which CGRP down-regulates 3-OS HS remains to be elucidated, it is possible that CGRP acts by interfering with the generation of 3-OS HS. For instance, HS is transformed by 3-O sulfotransferase (3-OST) to yield 3-OS HS[46], and CGRP could modulate 3-OST activity or its substrates. Other studies should also determine how CGRP interferes with the endolysosomal machinery and abrogates the need for low pH during HSV-1 infection, which might also be related to our previous results showing that CGRP diverts HIV-1 degradation away from endolysosomes[19].

For HSV-2, we found that infection of MDLCs occurs via langerin-mediated endocytosis in a pH-independent manner. HSV-2 is therefore probably internalized following its binding to langerin, and could be delivered to endocytic compartments and/or Birbeck granules, i.e., langerin-formed intracellular structures that are unique to LCs and are part of the endosomal recycling machinery[36]. As our previous studies showed that CGRP has no effect on langerin internalization[19], CGRP-mediated inhibition of HSV-2 infection probably occurs in a pre-entry step. Indeed, compared with langerin homo-trimers that are essential for efficient ligand binding[20] (e.g., including large pathogens such as HSV), we speculate that CGRP-induced atypical langerin double

trimers might be less efficient, and even defective, in ligand binding. Structural studies could reveal whether isolated langerin double trimers have steric interference for HSV-2 binding, leading to decreased HSV-2 internalization and infection.

Based on our results, we propose an original preventive neuro-immune approach, namely using CGRP-based formulations for inhibition of HSV-1/2 infection. As epidemiological and molecular studies indicate a synergistic relationship between HSV-2 and HIV-1 during co-infection[47], future CGRP-based formulations might represent a cost-effective prevention strategy, i.e. by inhibiting LCs-mediated infection with both HSV and HIV-1 simultaneously. Collectively, our previous studies and the results reported herein reinforce the anti-viral role and potential utility of CGRP, in the neuro-immune control of mucosal viral infections.

METHODS

Ethical statement

The study was performed under local ethical approval (Comité de Protection des Personnes CPP Paris-IdF XI, N.11016) and all patients signed informed consents.

Viruses

HSV-1 and HSV-2 primary isolates were derived from genital lesions of HSV^+ patients and were obtained from the Virology Service at the Cochin Hospital, Paris, France. All primary isolates were amplified once in Vero cells, titrated via standard plaque assay, aliquoted and stored at −80 °C.

MDLCs and inner foreskin LCs

Peripheral blood mononuclear cells (PBMCs) from healthy HIV-1 seronegative individuals, $CD14^+$ monocytes and MDLCs were separated as we previously described[29].

Normal foreskin tissues were derived from healthy adults undergoing circumcision and were obtained from the Urology Service at the Cochin Hospital, Paris, France. Inner foreskin epidermal cell suspensions were prepared using enzymatic digestion with dispase/trypsin, as we described[15,18]. For receptor blocking experiments, suspensions were enriched for immune cells using Ficoll gradient.

Flow cytometry

To characterize their phenotype or expression of HSV entry receptors, MDLCs or inner foreskin epidermal cells ($0.5\text{-}1 \times 10^5$/well, in duplicates in round bottom 96-wells plate) were stained for 20 min on ice in a final volume of 50 µl phosphate-buffered saline (PBS) with the Abs indicated in Supplementary Table 1. Matched isotype control Abs were included for HSV entry receptors experiments and fluorescent profiles were acquired using a Guava easyCite and analyzed with the InCyte software (Merck-Millipore).

HSV infection and apoptosis/viability

MDLCs or inner foreskin epidermal cell suspensions (0.5×10^5 MDLCs and 2×10^5 epidermal cells / 200 µl/ well, in duplicates in round bottom 96-wells plates) were re-suspended in complete RPMI medium[29]. Cells were either left untreated or pre-treated for 24 h at 37 °C with the indicated molar concentrations of CGRP (AnaSpec), or the metabolically stable CGRP analogue SAX we described and prepared[39,40]. The cells were then re-suspended in RPMI medium without serum and pulsed for 2 h (MDLCs) or 24 h (inner foreskin epidermal cells) at 37 °C with primary isolates of either HSV-1 or HSV-2 at the indicated MOIs. In some experiments with MDLCs, the following inhibitors (Sigma) were included: NH_4Cl (10 mM), bafilomycin A1 (10 nM), dynasore (100 µM), methyl beta cyclodextrin (10 mM). Of note, at the concentrations / time point tested, none of the agonists and inhibitors affected MDLCs viability, as evaluated below. The medium-containing virus was then removed, the cells were washed and further incubated at 37 °C in complete RPMI medium. At 24 and 48 h pi, cells were washed with 0.1 M glycine buffer pH = 2.0 for 2 min at room temperature in order to detach non-internalized HSV virions. Cells were then surface-stained for langerin expression as above, fixed with PBS / 4% paraformaldehyde (PFA, Electron Microscopy Sciences), washed, permeabilized with PBS / 0.1% saponin, and intracellularly stained for 20 min at room temperature for HSV-1/2-gD expression (see Supplementary Table 1). Cells were then analyzed by flow cytometry as above. Apoptosis and viability of non-infected and HSV-infected MDLCs were evaluated by Annexin / PI staining using an Annexin V-FITC kit or Viobility Fixable Dye staining (Miltenyi Biotec), according to the manufacturer's instructions.

HSV receptors blocking assay

MDLCs or inner foreskin epidermal cell suspensions that were enriched for immune cells (0.5×10^5 cells/well, in duplicates in round bottom 96-wells plate) were pre-incubated for 1 h at 37 °C in a final volume of 25 µl PBS with the nAbs indicated in Supplementary Table 1. Of note, we verified that the langerin nAb does not interfere with langerin detection using the DCGM4 clone (data not shown). Cells were also incubated for 1 h at 37 °C with 10U/ml of heparinase II and/or II (from *Flavobacterium heparinum*; Sigma). Next, cells were washed and infected with HSV-1 or HSV-2, as above.

Cross-linking and Western blot

MDLCs (2.5×10^6 cells / 5 ml / T25 flasks) were either left untreated or treated for 24 h at 37 °C with 1 µM CGRP or SAX. Cells were then cross-linked as previously described[20], using the chemical cross linker disuccinimidyl glutamarate (DSG, ChemCruz) that was dissolved to 25 mM in dimethyl sulfoxide (DMSO) and added to the suspensions at 1 mM for 30 min at room temperature. Samples (25 µg protein / sample) were run over a 10% SDS-PAGE, transferred onto nitrocellulose membranes at 4 °C, and membranes were blocked with blocking buffer (Tris-buffered saline / 0.5% Tween 20/0.5% dry milk) for 1 h at room temperature with constant agitation. Membranes were next incubated overnight at 4 °C with 0.1 µg/ml goat polyclonal anti-human langerin IgG Ab (R&D Systems), followed by 0.5 µg/ml horse-radish-peroxidase-conjugated donkey anti-goat polyclonal Ab (Promega), diluted in blocking buffer. Revelation was performed with ECL-Prime chemiluminescence detection kit (Amersham). Images were acquired with the Fusion FX camera platform (Vilber Lournmat) and expression quantified with the ImageJ software.

Statistical analysis

Statistical significance was analyzed by two-tailed Student's t-test or ANOVA. The relationships between HSV infection and replication were analyzed with Pearson correlations.

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AUTHOR CONTRIBUTIONS

E.C., J.M., and Y.G. performed the experiments; F.R. provided primary HSV isolates; A.S. prepared and provided SAX; T.H.vK prepared and provided clone HS4C3 against 3-OS HS; N.B.D. and M.Z. provided foreskin tissues; Y.G. and M.B. designed the experiments; Y.G. conceived the study and wrote the paper.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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