Kaposi's-sarcoma-associated-herpesvirus-activated dendritic cells promote HIV-1 trans-infection and suppress CD4⁺ T cell proliferation

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Infection of Kaposi's sarcoma-associated herpesvirus (KSHV) is commonly occurred in AIDS patients. KSHV and HIV-1 act cooperatively in regulating infection with each other and in human carcinogenesis. Dendritic cells (DCs), as the pivotal cells in host immunity, may be modulated by both viruses, for immunoevasion and dissemination, therefore, the interaction between DCs and each virus has been a prior focus for pathogenesis elucidation. Here, we assessed the potential effect of KSHV on DC–HIV–1 interaction. We found that KSHV stimulation could promote maturation of monocyte-derived DCs (MDDCs) and impaired the ability of MDDCs to drive proliferation of resting CD4⁺ T cells, demonstrating the immunosuppression induced by KSHV. More importantly, KSHV-stimulated MDDCs could capture more HIV-1 and efficiently transferred these infectious viruses to Hut/CCR5 T cell line. Our results reveal the novel modulation of DC-mediated HIV-1 dissemination by KSHV, and highlight the importance of studying DC–HIV–1 interaction to elucidate HIV/AIDS pathogenesis.

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Introduction

The deterioration of the host immune system caused by HIV-1 infection leads to lack of control of microbial translocation (Brenchley et al., 2006). As a result, AIDS-associated opportunistic pathogenic fungi, bacteria and viruses exacerbate deterioration of the immune response and accelerate AIDS progression (Qin et al., 2011, 2012). Understanding the immune deterioration induced by these co-pathogens could facilitate our understanding of HIV/AIDS pathogenesis.

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus (HHV)-8, is of particular concern when infecting immunosuppressed individuals, including AIDS patients (da Silva and de Oliveira, 2011; Lennette et al., 2005). KSHV infection is associated with multiple human malignancies (Dai et al., 2012; Sakakibara and Tosato, 2011). In AIDS patients, KSHV infection commonly causes Kaposi's sarcoma (KS) (Ahmed et al., 2012; Armstrong et al., 2013; Restrepo and Ocazionez, 2011). It is noteworthy that KSHV and HIV-1 co-operate in human carcinogenesis and viral pathogenesis (Bovin et al., 2000; da Silva and de Oliveira, 2011), although the co-infection or co-localization of both viruses seems not critical for their co-operation (Caselli et al., 2005; Mercader et al., 2001). HIV-1-induced immune impairment likely contributes to the immunoevasion of herpesviruses and significantly increases human carcinogenesis. In contrast, increased HIV-1 replication has been observed in CD4⁺ T cells and monocytes when co-cultured with KSHV-infected cells (Caselli et al., 2005; Mercader et al., 2001).

Dendritic cells (DCs) are pivotal immune cells for regulating host innate and adaptive immunity. DCs and mononuclear cells may be targeted by both KSHV and HIV-1 for infection (Rappocciolo et al., 2006; West et al., 2011), although these two viruses show a big difference in cellular tropism. Importantly, both viruses can modulate DC function, and the numerical and functional alterations of DCs induced by either virus may represent an important strategy for escaping from the host immune response. KS patients show significantly lower frequency of myeloid and plasmacytoid DCs, and this reduction is greater with advanced stages of disease (Della Bella et al., 2006). Impairment of DC function by KSHV has been reported in vitro studies and in vivo investigations of tumor-bearing animals and cancer patients with solid or hematological malignancies, and DCs display significant defective antigen uptake and presentation and allostimulatory ability (Cirone et al., 2012, 2008, 2007; Rappocciolo et al., 2006; West et al., 2011). KSHV-infected DCs have impaired production of interleukin (IL)-12 (Della Bella et al., 2006), and skewed cytokine production towards induction of a T helper (Th)2 response (Hensler et al., 2009). KSHV also interferes with DC differentiation from CD34⁺ and monocytes precursors (Cirone et al., 2008, 2007). DC function is also modulated by HIV-1, and the interaction of DCs and HIV-1 plays a critical role in viral pathogenesis (Wu, 2008; Wu and KewalRamani, 2006).
Much effort has been made to understand the co-operation between HIV-1 and KSHV in regulating viral replication and human carcinogenesis, while limited investigation has focused on the potential effects of KSHV on DC–HIV-1 interactions. Here, we report that KSHV stimulation could promote maturation of monocyte-derived DCs (MDDCs), and KSHV-stimulated MDDCs could capture more HIV-1 particles and efficiently transfer these infectious viruses to CD4⁺ T cell line Hut/CCR5. Our results reveal the novel modulation of KSHV and DC-mediated HIV-1 dissemination. Understanding the effects of co-operating pathogens on DC–HIV-1 interaction could facilitate the elucidation of HIV/AIDS pathogenesis.

Results

Induction of infectious KSHV from latently infected endothelial cells

iSLK.219 cell displays tight control of KSHV latency and is efficiently inducible by doxycycline. GFP is constitutively expressed in these cells and RFP is expressed during lytic replication, as described previously (Myoung and Ganem, 2011). Doxycycline induces iSLK.219 cells for lytic replication to generate large quantities of infectious rKSHV.219 viruses (Fig. 1A). Infection of HEK293T cells was quantified (Fig. 1B) by detecting GFP expression with flow cytometry, and a dose-dependent viral infection was observed. These infectious rKSHV.219 viruses were killed by ultraviolet (UV) irradiation for 4 h (Fig. 1B). The replication of rKSHV.219 in HEK293T cells was confirmed by detecting the mRNA production of rKSHV ORF8 and LANA genes. ORF8 and LANA represent lytic and latent gene products of KSHV, respectively. Viral gene expression could be detected for high expression in KSHV-infected HEK293T cells at the indicated time of post-infection, but no viral gene expression was observe when UV-killed KSHV for infection (Fig. 1C). Together, these data demonstrate infectious recombinant KSHV viruses can be induced from latently infected iSLK.219 cells.

Stimulation of rKSHV promotes maturation of MDDCs

We and others have previously proved that AIDS-associated pathogens can promote maturation of DCs (Diou et al., 2010a, 2010b; Qin et al., 2011, 2012; Reuter et al., 2010; Vachot et al., 2008). To determine the potential effects on MDDC maturation by KSHV, immature MDDCs were co-cultured with different amounts of rKSHV for 48 h. Cell phenotype was monitored by immunostaining of surface markers. The co-stimulatory molecules of CD83 and CD86 and HLA-DR showed enhanced expression (Fig. 2A), indicating maturation of MDDCs. Lipopolysaccharide (LPS) was used as the control for inducing MDDC maturation (Wang et al., 2007). When rKSHV was killed by UV radiation, the inactivated rKSHV could still promote expression of CD83, CD86

Fig. 1. Generation of infectious recombinant KSHV stock. (A) RTA-inducible SLK cells (iSLK.219) harboring recombinant KSHV (rKSHV.219) were induced by 0.2 μg/ml doxycycline for 48 h. All cells remained GFP⁺ RFP⁻ when no induction stimulus was provided, whereas in response to doxycycline induction, > 70% of cells become RFP⁺, suggesting that lytic replication was highly induced. (B) and (C) 293T cells were infected for 48 h with different amounts of infectious units of rKSHV, and GFP⁺ cells were monitored by flow cytometry. rKSHV was killed by UV irradiation for 4 h. Percentage of GFP⁺ cells are shown. (C) The relative mRNA production of rKSHV ORF8 and LANA genes was quantified and normalized with β-actin. KSHV-w/o RT, means these RNA samples isolated from KSHV-infected cells were not reverse-transcribed. Results of one representative experiment out of three are shown. ***P < 0.001, were considered significant difference in Student’s t test.
and HLA-DR, suggesting that viral binding can induce cell maturation, and the apparent viral gene expression is dispensable (Fig. 2A). To confirm this point, rKSHV replication in MDDCs was measured by detecting the mRNA production of rKSHV ORF8 and LANA genes. There was no or slight enhancement of mRNA production at 2 or 4 days post-viral inoculation (Fig. 2B),
emphasizing that rKSHV-induced MDDC maturation was not attributed to intracellular replication of viruses. Cell viability after 48 h viral inoculation was monitored by PI exclusion assay. The lower amount of rKSHV (MOI 0.1 or 0.5) that was used above for DC stimulation caused <1.5% cell death, whereas the higher amount of virus (MOI 1) led to about 20% cell death. H2O2 (3%) was used as a control for necrosis induction (Fig. 2C). Taken together, these data demonstrated that the stimulation of rKSHV promotes maturation of MDDCs, and viral gene expression may be dispensable for MDDCs maturation.

**rKSHV stimulation impairs DC-induced proliferation of CD4+ T cells**

KSHV has been reported for antagonizing host antiviral responses by suppresses activation of interferon regulatory factor, contributing to immune-evasion (Li et al., 2012; Zhu et al., 2002). To investigate the potential effect of KSHV on DC-induced T-cell proliferation, MDDCs were pulsed with rKSHV or control medium for 48 h. After washing, MDDCs were co-cultured with CFSE-labeled, allogeneic resting CD4+ T cells for an additional 6 days (Fig. 3A), and T-cell proliferation was monitored by detection of CFSE intensity. rKSHV-activated DCs impaired CD4+ T-cell proliferation (Fig. 3B). KSHV-inoculated MDDCs were investigated for viral constitutively expressed-GFP to make sure no GFP color background has been contaminated in CFSE detection (Fig. 3C).

**rKSHV-stimulated MDDCs enhance HIV-1 endocytosis in a DC-SIGN-independent manner but impair HIV-1 infection**

Mature DCs take up HIV-1 particles, which can be sequestered intracellularly for escaping proteolysis and transferred to CD4+ T cells (Wang et al., 2007; Wu and KewalRamani, 2006). To determine whether rKSHV-stimulated MDDCs could also take up more HIV-1 particles, the non-infectious VLP, HIV-gag-GFP/JFRL, was used, and VLP binding and internalization were tested. At 4°C, more VLPs were bound to rKSHV-stimulated MDDCs, and GFP-positive cells increased from 5.8% to 7.2–10.9%, as detected by flow cytometry (Fig. 4A). At 37°C, rKSHV-stimulated MDDCs took up many more VLPs compared with medium-treated MDDCs, and the GFP-positive cells increased from 16.3% to >64% (Fig. 4A). Notably, most of the VLPs could not be removed with trypsin digestion from the cell surface of rKSHV-stimulated MDDCs, suggesting viral internalization. Immature-DC-mediated viral uptake mainly depends on viral surface binding (Wang et al., 2007), as expected, VLPs could be removed with trypsin digestion from the surface of immature MDDCs (Fig. 4A). Similarly, confocal microscopy showed that more VLPs were internalized in rKSHV-stimulated MDDCs, however, medium-treated MDDCs bound VLPs mainly on the cell surface (Fig. 4B).

Next, we were interested in whether the enhanced HIV-1 binding and endocytosis could promote HIV-1 infection. Different amounts of infectious rKSHV were used to stimulate MDDCs for 48 h, and MDDCs were inoculated with single-cycle, luciferase reporter HIV-luc/JFRL (CCR5-tropic), and HIV-1 infection was measured by detecting the luciferase activity in cell lysates 5 days post-infection. HIV-1 infection in rKSHV-stimulated MDDCs was impaired (Fig. 4C).

The molecule of DC-SIGN has been shown to be hijacked by HIV-1 for mediating viral transmission to CD4+ T cells for robust replication (McDonald et al., 2003; Wang et al., 2007, 2009; Wu, 2008; Wu and KewalRamani, 2006). To investigate whether rKSHV-activated MDDCs can promote HIV-1 transfer to T cells, MDDCs stimulated with different amounts of rKSHV or medium were loaded with HIV-luc/JFRL, and then co-cultured with Hut/CCR5 cells, the CD4+ T cell line (Fig. 6A). Viral transfer was measured by testing viral infection in T cells. rKSHV-activated MDDCs enhanced HIV-1 transfer to T cells (Fig. 6B).

DC and T cells form the virological synapses for providing the most efficient route for HIV-1 transfer (McDonald et al., 2003; Wang et al., 2007), and ICAM-1-lymphocyte function-associated antigen (LFA)-1 interaction is involved in the formation of DC-T-cell conjugation (Wang et al., 2009). To investigate whether rKSHV-activated MDDCs could increase ICAM-1 expression and promote HIV-1 transfer to T cells, ICAM-1 was immunostained and detected by flow cytometry. Stimulation with rKSHV enhanced ICAM-1 expression on MDDCs by 1.4–1.7-fold, regardless of whether infectious or inactivated rKSHV was used (Fig. 6C). The formation of virological synapses was visualized by confocal microscopy, and Hut/CCR5 T cells were used as target cells (Fig. 6D). More viral particles were efficiently recruited at rKSHV-activated MDDC-T cell contact sites to form the virological synapses.

Taken together, these data demonstrate that rKSHV-stimulated MDDCs enhance ICAM-1 expression and recruit more HIV-1 particles to the virological synapses for enhanced viral transmission.

**Discussion**

DCs are pivotal immune cells for regulating host immunity (Wu, 2008; Wu and KewalRamani, 2006). The multiple functions of DCs have been modulated by both KSHV and HIV-1 for regulating viral dissemination and immunoevasion. The susceptibility of DCs for both KSHV and HIV-1 infection provides a converging point for investigating the interaction between KSHV and HIV-1. The abundant expression of DC-SIGN offers KSHV binding and infection of monocyes-derived DCs and macrophages (Rappocciolo et al., 2006). KSHV infection can decrease DC-SIGN expression (Rappocciolo et al., 2006), and inhibition of its transcription has been reported in DCs infected by HHV-6 (Niiya et al., 2004). We also found that inoculation of UV-inactivated KSHV compromised, but not completely abolished, downregulation of DC-SIGN, indicating that viral binding and replication might both modulate DC-SIGN expression (data not shown). KSHV infection promotes maturation of DCs (Rappocciolo et al., 2006), as assessed by upregulation of co-stimulatory molecules CD80 and CD83. We found that KSHV binding also promoted maturation of DCs, although the viral components
Fig. 3. rKSHV stimulation impairs DC-induced proliferation of CD4⁺ T cells. MDDCs were pulsed with rKSHV or control medium for 48 h. After washing, MDDCs were co-cultured with CFSE-labeled, allogeneic resting CD4⁺ T cells for an additional 6 days, as shown schematic illustration (A). (B) CFSE-labeled T cells were gated and proliferation was monitored by detection of CFSE intensity. (C) KSHV-inoculated MDDCs were investigated for viral constitutively expressed-GFP to make sure no GFP color background has been contaminated in CFSE detection as in (B). MFI is calculated with percentage timing fluorescence value. Results of three independent donors are shown. Positive percentage and MFI are indicated.
Fig. 4. rKSHV-stimulated MDDCs can endocytose more HIV-1 particles. MDDCs were stimulated with rKSHV for 48 h, HIV-1 VLPs (4 ng p24gag) were added for 2 h incubation at 4 or 37 °C, and some samples were treated with trypsin for 5 min to remove surface-bound viruses. Gag-GFP level was detected by flow cytometry, and the positive percentages and the calculated MFI values from one representative of six experiments are shown. MFI is calculated with percentage timing fluorescence value. (B) Enhanced internalization of HIV-1 VLPs in rKSHV-stimulated DCs was observed under confocal microscopy. MDDCs were treated with rKSHV (MOI 0.5) and pulsed with HIV-1 VLPs as described for (A), MDDCs were fixed and immunostained for β-actin, nuclei were stained by DAPI, and cells were observed by confocal microscopy. Scale bars, 5 μm. (C) MDDCs were treated with rKSHV for 48 h, and incubated with single-cycle luciferase reporter virus HIV-Luc/JRFL (5 ng p24gag) for 2 h. After washing, HIV-1-pulsed MDDCs were incubated for 5 days, and HIV-1 infection was detected by measuring the luciferase activity in cell lysates. Results of one representative experiment out of four are shown. **P < 0.01 and ***P < 0.001, were considered significant difference in Student’s t test. Data are mean ± standard deviation (SD); cps, counts per second.
accounting for maturation induction need to be identified. KSHV infection has been shown to alter chemokine receptor CCR6 expression on immature DCs, inhibit the migratory behavior of both immature and mature DCs, and induce cytoskeleton reorganization (Cirone et al., 2012). KSHV-treated DCs show impaired antigen presentation and allostimulatory activity, and alter the cytokine production profiles (Cirone et al., 2008; Della Bella et al., 2006; Hensler et al., 2009; Rappocciolo et al., 2006). We confirmed the defective capacity of KSHV-stimulated DCs for inducing proliferation of resting CD4$^+$ T cells. Our data also suggest that robust infection of KSHV may not be necessarily required for DC functional impairment. In contrast, DC maturation induced by KSHV-encoded components has been exploited for improving the efficacy of vaccine vectors (Rowe et al., 2009). The gene encoding KSHV vFLIP (viral encoded, Fas-associated death domain-like IL-1β-converting enzyme inhibitory protein) has been incorporated into a lentivector (Rowe et al., 2009).

Expression of this oncoprotein activates nuclear factor-κB in mouse bone-marrow-derived DCs and promotes maturation of DCs; the co-stimulatory markers CD80, CD86, CD40, and ICAM-1 are upregulated; and tumor necrosis factor α and IL-12 are secreted. The vFLIP-expressing lentivector increases CD8$^+$ T-cell response, correlated with improved tumor-free survival in a tumor therapy model, and reduces the parasite load when mice are challenged with Leishmania donovani.

The alteration of DC function induced by KSHV will surely provide more opportunities for HIV-1 hijacking and further deterioration of the immune system. We have shown previously that AIDS-associated fungal pathogens enhance maturation of DCs and promote DC ability to mediate HIV-1 transmission (Qin et al., 2011, 2012). Here, we demonstrated that KSHV-stimulated DCs also could capture more HIV-1 particles and enhanced HIV-1 transfer to CD4$^+$ T cells for robust infection. The increased expression of ICAM-1 strengthens the DC-T cell interaction, which
is a key route for HIV-1 transfer between cells. DCs are not the only hijacked target for KSHV to enhance HIV-1 replication and viral burden. Several KSHV proteins, for example, LANA, the immediate-early protein KIE-2, the oncoprotein vFLIP K13, and ORF50 (Rta), have been reported to lead to activation of HIV-1 LTR to enhance HIV-1 transcription in CD4+ T cells (Caselli et al., 2003a, 2003b, 2001; Sun et al., 2005). KSHV infection induces HIV-1 reactivation in chronically infected cell lines and in PBMCs from asymptomatic patients (Caselli et al., 2005). In contrast, HIV-1-induced immunosuppression plays an important role in the pathogenesis of KSHV-associated cancer. HIV-1 Tat protein activates lytic cycle replication of KSHV (Aoki and Tosato, 2004; Merat et al., 2002; Varthakavi et al., 2002; Zeng et al., 2007), and induces KS-like lesions in nude mice and angiogenesis. HIV-1 Tat protein can increase KSHV vGPCR (KSHV-encoded G-protein-coupled receptor) signaling, resulting in the KSHV lytic biological cycle and accelerated tumorigenesis (Patel et al., 2003).

Besides KSHV, DCs also can be targeted by other AIDS-associated opportunistic pathogens to cause deterioration of the host immune response and promote HIV-1 dissemination (Diou et al., 2010a, 2010b; Qin et al., 2011, 2012; Reuter et al., 2010; Vachot et al., 2008). Give the pivotal role of DCs in HIV-1

Fig. 6. rKSHV-stimulated MDDCs increase surface expression of ICAM-1 and facilitate MDDC-mediated HIV-1 transmission. (A) Schematic illustration for DCs treatment by KSHV and HIV-1 trans-infection mediated by DCs. (B) Enhanced HIV-1 transmission mediated by rKSHV-stimulated MDDCs. HIV-luc/JFRL-loaded, rKSHV- or medium-stimulated MDDCs were co-cultured with Hut/CCR5 for 3 days, and HIV-1 transfer was detected by measuring viral infection. DC alone, means that HIV-loaded MDDC are not co-cultured with Hut/CCR5 T cells. Data are mean ± SD. Results of one representative experiment out of four are shown. *P < 0.05 and **P < 0.01 were considered significant difference in Student’s t test. (C) Increased ICAM-1 expression on rKSHV-stimulated MDDCs. MDDCs were treated with rKSHV, and the surface level of ICAM-1 was detected by flow cytometry. The MFI values are shown, MFI is calculated with percentage timing fluorescence value. (D) Enhanced viral concentration on the contact sites between MDDCs and T cells. MDDCs were treated with rKSHV (MOI 0.5) for 48 h, and HIV-1 VLPs (4 ng p24gag) were added for 2 h incubation, and after washing Hut/CCR5 cells were added as target cells for an additional 30 min co-culture. Cells were fixed for immunostaining or labeling and observed by confocal microscopy. β-Actin was displayed in red, and nuclei were stained blue with DAPI. DIC, differential interference contrast. Scale bars, 5 μm.
pathogenesis, understanding the effects of these co-pathogens on DC–HIV-1 interaction could help to elucidate HIV-1 pathogenesis and the search for novel strategies to combat AIDS.

Materials and methods

Cells

Human peripheral blood mononuclear cells (PBMCs) from healthy donors were provided by the Blood Center of Shanghai, China. CD14+ monocytes and resting CD4+ T cells were purified from PBMCs using magnetic beads (BD Biosciences) as described previously (Shen et al., 2012). CD14+ monocytes were cultured with granulocyte–macrophage colony-stimulating factor and IL-4 for 6 days to generate immature DCs. The human embryonic kidney cell lines HEK293T and the CD4+ T-cell line Hut/CCR5 were kind gifts from Dr. Li Wu (the Ohio State University, USA) and Dr. Vineet Kewal Ramani (National Cancer Institute, NIH, USA).

The KSHV latently infected SLK endothelial cells, iSLK.219, have been described previously (Myoung and Ganem, 2011), iSLK.219 cells were derived from SLK endothelial cells that were first engineered with a doxycycline-inducible RTA transgene and then latently infected with a recombinant KSHV.219 virus. This cell line was donated by Dr. Myoung J (Howard Hughes Medical Institute, University of California, San Francisco, CA, USA). iSLK.219 cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with hygromycin (100 μg/ml) (Invitrogen), puromycin (4 μg/ml) (Invitrogen) and G418 (100 μg/ml) (Sigma), 10% fetal bovine serum, and penicillin–streptomycin.

Flow cytometry

Cells were stained with specific monoclonal antibodies (McAbs) or isotype-matched IgG controls. McAbs against the following human molecules were used for staining (clone numbers and resources are given in parentheses): APC-Alexa Fluor750 CD11c (B-ly6; BD Pharmingen), Phycoerythrin (PE)-ICAM-1 (CD54) (HA58; eBioscience), PE-CD83 (HB15e; eBioscience), PE-CD86 (IT2.2; eBioscience), PE-DC-SIGN (DC-specific ICAM-3-grabbing nonintegrin) (ebh209; eBioscience), and APC-cy7-HLA-DR (LN3; eBioscience). Stained cells were detected with an LSRII flow cytometer (BD Biosciences Pharmingen) and analyzed with FlowJo 7.6.1 software (TreeStar Inc.).

rKSHV.219 generation and infection

Inducible iSLK.219 cells were generated as described previously (Myoung and Ganem, 2011). iSLK.219 cells contained recombinant KSHV.219 (rKSHV.219) and expressed RTA (replication and transcription activator) under control of a doxycycline-responsive promoter (tetracycline-responsive element). iSLK.219 cells were induced with 0.2 μg/ml doxycycline. Thirty-six hours post-induction, the medium was refreshed for an additional 3 days culture, and the supernatants containing rKSHV were harvested, filtered, aliquoted and stored at −80°C. KSHV was inactivated for 30 min. Cells were seeded on the poly-L-lysine-coated microscope slides for 6 days to generate immature DCs. The human embryonic kidney cell lines HEK293T and the CD4+ T-cell line Hut/CCR5 were kind gifts from Dr. Li Wu (the Ohio State University, USA). Virus stocks and VLPs were quantified using p24Gag capture ELISA and stored at −80°C.

HIV-1 binding and internalization

HIV-1 binding and internalization were quantified by flow cytometry using the VLP HIV-Gag-GFP/JRFL. KSHV-stimulated MDDCs were incubated with VLPs (4 ng p24Gag) for 2 h at 4 or 37°C, and washed. Some samples were prior-blocked with anti-DC-SIGN specific antibodies (10 μg/ml) (Clone 120507; Abcam) for 30 min. Some cells were treated with 0.25% trypsin (without EDTA) for 5 min at 37°C to remove surface-bound VLPs. The amounts of VLPs associated with MDDCs were quantified by flow cytometry.

HIV transmission assays

The luciferase reporter system was adopted for assay of HIV-1 transmission as previously described (Qin et al., 2011, 2012). MDDCs were pulsed with 5 ng p24Gag amounts of pseudotyped HIV-luc/JRFL for 2 h, and cells were washed for culture or co-culture with CD4+ T cell line, Hut/CCR5 cells. Cells were harvested at 3 days post-infection, and the cell lysates were measured for luciferase activity with a commercially available kit (Promega).

Confocal microscopy

The formation of virological synapses was observed by confocal microscopy. MDDCs were pulsed with HIV-1 VLPs (4 ng p24Gag of HIV-Gag-GFP/JRFL) and co-cultured with Hut/CCR5 for 30 min. Cells were seeded on the poly-L-lysine-coated microscope slides and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 1 h at 4°C. Cells were immunostained with anti-β-actin antibodies (AC-15; Sigma), followed by staining with Alexa-Fluor-555-labeled goat anti-mouse IgG (Invitrogen). Nuclei were stained with DAPI. Slides were mounted with Fluorescent Mounting Medium (Dako) and observed using a laser scanning confocal microscope (Leica SP5).

T-cells proliferation assay

KSHV-stimulated MDDCs were used to co-culture with CFSE (5-carboxyfluorescein diacetate succinimidyl ester) (Invitrogen)-labeled allogeneic resting CD4+ T cells for 6 days at the ratio of 1:10 (MDDC:T). The T cells were gated based on the CFSE-positive population, and proliferation was analyzed using a flow cytometer by detecting CSFE intensity.
Statistical analysis

Statistical analysis was performed using paired t test with the SigmaStat program.

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J. Wu et al. / Virology 440 (2013) 150–159


J. Wu et al. / Virology 440 (2013) 150–159


