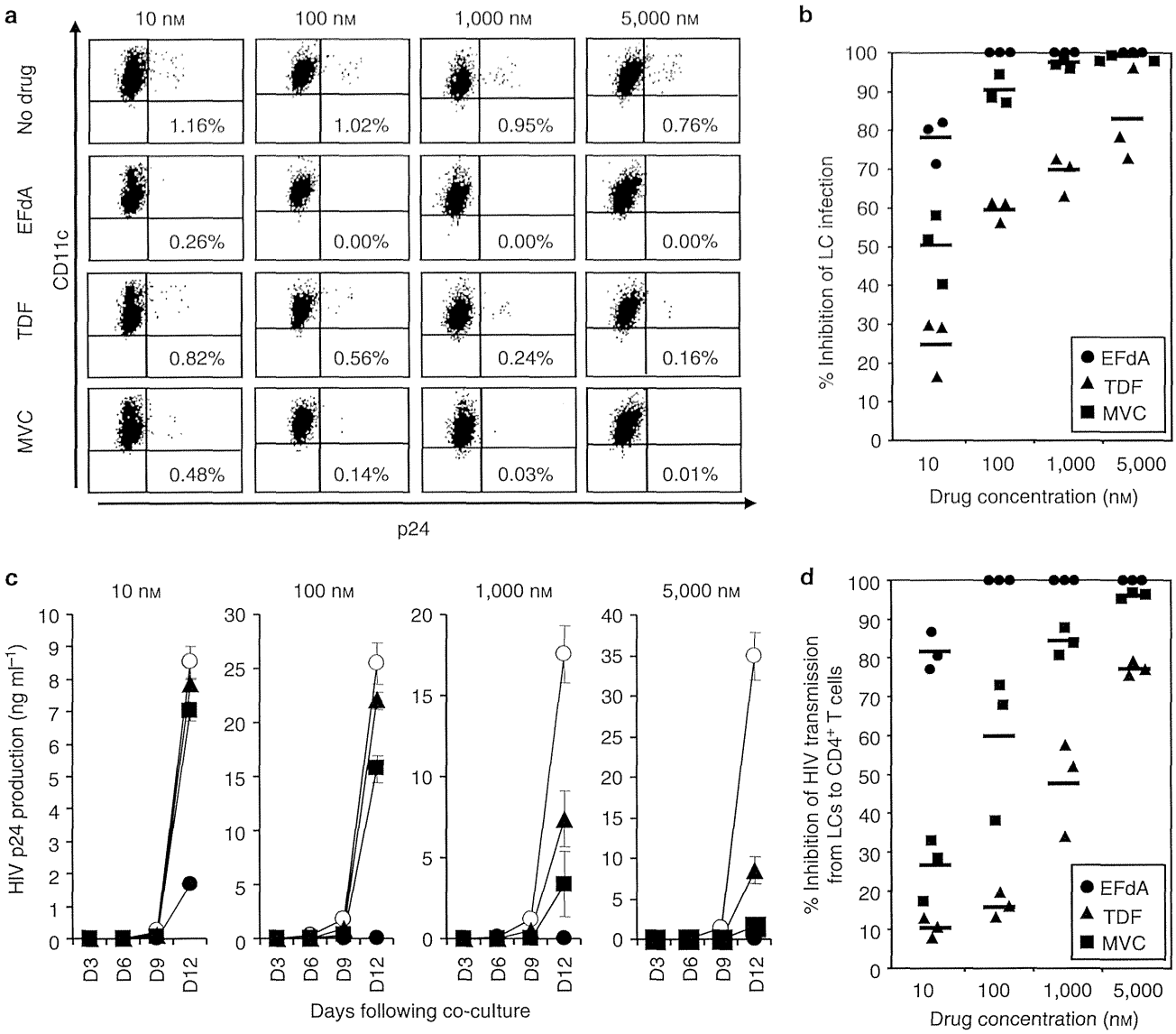


Figure 1. Preincubation of skin explants with EFdA blocks R5-HIV-1 infection in LCs and subsequent virus transmission to cocultured CD4$^+$ T cells. LCs within skin explants were preincubated with no drug (○) or the indicated concentrations of EFdA (●), TDF (▲), and MVC (■) for 30 minutes, exposed to HIV-$1_{Ba\text{-}L}$ for 2 hours, and then floated on culture medium to allow migration of LCs from the explants. Emigrating cells from the epidermal sheets were collected 3 days following HIV-1 exposure. HIV-1-infected LCs were assessed by HIV-1 p24 intracellular staining in langerin$^+$ CD11c$^+$ LCs (**a**, **b**), or further cocultured with autologous CD4$^+$ T cells and culture supernatants were assessed for p24 content by ELISA on the indicated days (**c**, **d**). Summary of percent inhibition of LC infection (**b**) and virus transmission to CD4$^+$ T cells (**d**) of 12 experiments using skin explants from 12 individuals with the indicated each concentration of EFdA (●), TDF (▲), and MVC (■) are shown. Mean values obtained from different donors are shown as horizontal marks (**b**, **d**). EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LCs, Langerhans cells; MVC, maraviroc; TDF, tenofovir.

Intriguingly, even in 1–3 days following the removal of EFdA (1,000 nM), EFdA completely blocked HIV-1 infection of mLCs as well as subsequent virus transmission from mLCs to cocultured CD4$^+$ T cells, whereas TDF and MVC rapidly lost their anti-HIV-1 activity within days (Figure 2c–f). No cellular toxicity was noted for any of these drugs at the doses used in these experiments (Supplementary Figure S2 online). When similar experiments were conducted using peripheral blood mononuclear cell as target cells, virtually identical favorable persistency of EFdA in antiviral activity compared with that of TDF was observed (data not shown).

In the present work, we demonstrated that EFdA exerted extremely more potent anti-HIV-1 activity in LCs than did TDF and MVC, and the potent anti-HIV-1 activity of EFdA persisted for at least 3 days. Of note, the efficacy of TDF gel in CAPRISA 004 has been linked to its long intracellular half-life (Abdool Karim *et al.*, 2010; Rohan *et al.*, 2010). Our data strongly indicate that EFdA may serve as a promising microbicide to block sexual transmission of HIV-1 because of its potent anti-HIV-1 activity, low cytotoxicity, and superior

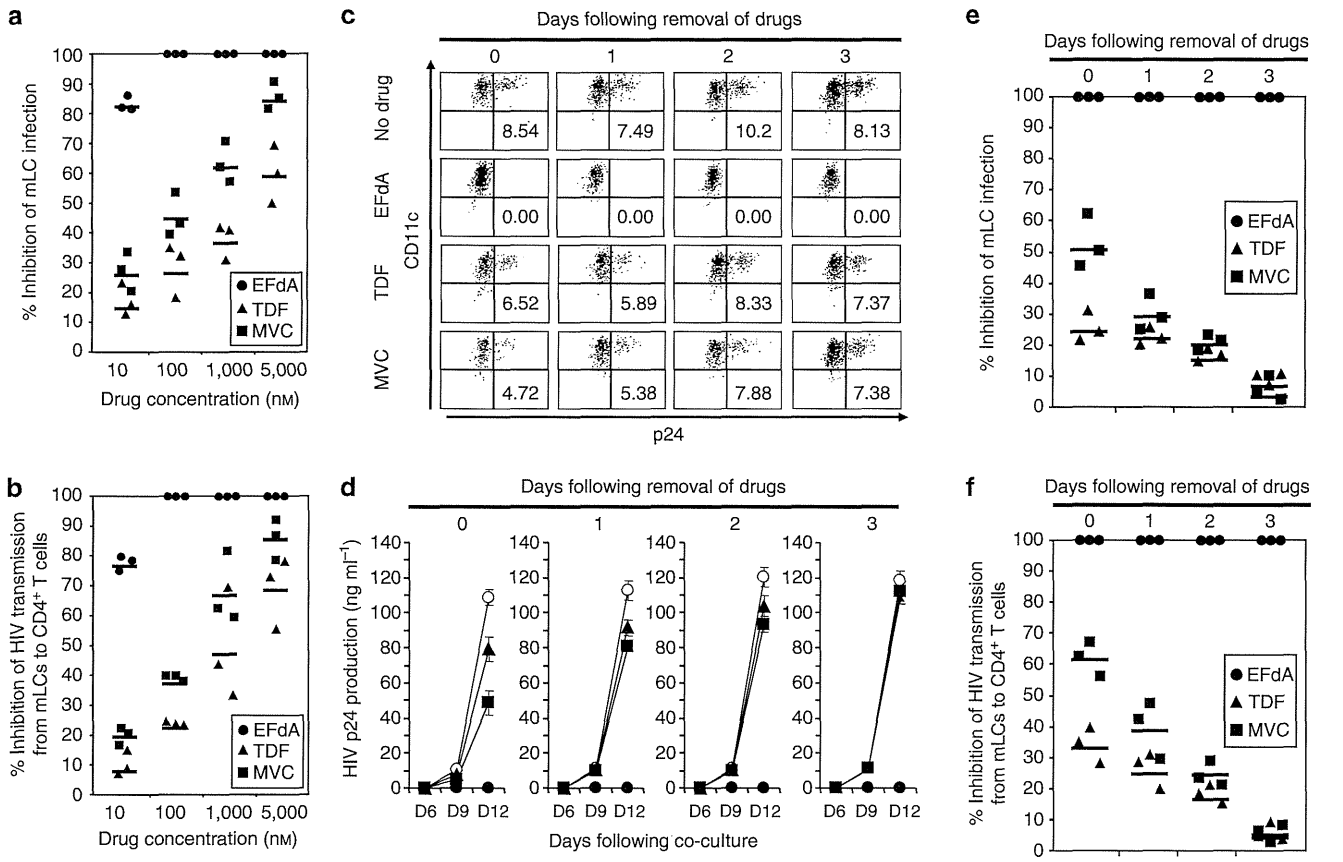


Figure 2. Preincubation of skin explants with EFdA blocks subsequent R5-HIV-1 infection in LC in a dose-dependent manner. mLCs were preincubated with no drug (○) or the indicated concentrations of EFdA (●), TDF (▲) and MVC (■) for 30 minutes, and then immediately exposed to HIV-1Ba-L for 2 hours (**a**, **b**), or thoroughly washed to remove the extracellular drug and further cultured for 1, 2, or 3 days prior to exposure to HIV-1Ba-L for 2 hours (**c**–**f**). After 7 days of HIV-1 exposure, HIV-1-infected mLCs were assessed by HIV-1 p24 intracellular staining in langerin$^+$ CD11c$^+$ mLCs (**a**, **c**, **e**), or further cocultured with autologous CD4$^+$ T cells and culture supernatants were assessed for p24 content by ELISA on the indicated days (**b**, **d**, **f**). Summary of percent inhibition of mLC infection (**a**, **e**) and virus transmission to CD4$^+$ T cells (**b**, **f**) of three independent experiments are shown. Mean values are shown as horizontal marks (**a**, **b**, **e**, **f**). EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LCs, Langerhans cells; mLCs, monocyte-derived LCs; MVC, maraviroc; TDF, tenofovir.

persistence of antiviral activity against HIV-1 in LCs.

CONFLICT OF INTEREST

HM is among coinventors on a patent for EFdA; all rights, title, and interest to the patent have been assigned to Yamasa Corporation, Chiba, Japan. The other authors state no conflict of interest.

ACKNOWLEDGMENTS

Financial support was provided by the Ministry of Health Science of the Japanese Government (201029002), the Intramural Research Program of Center for Cancer Research, National Cancer Institute, National Institutes of Health, and a grant for global education and research center aiming at the control of AIDS (Global Center of Excellence supported by Monbu-Kagakusho).

Takamitsu Matsuzawa[1], Tatsuyoshi Kawamura[1], Youichi Ogawa[1], Kenji Maeda[2], Hirotomo Nakata[3], Kohji Moriishi[4], Yoshio Koyanagi[5], Hiroyuki Gatanaga[6], Shinji Shimada[1] and Hiroaki Mitsuya[2,3]

[1]Faculty of Medicine, Department of Dermatology, University of Yamanashi, Yamanashi, Japan; [2]Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA; [3]Department of Infectious Diseases and Hematology, Kumamoto University School of Medicine, Kumamoto, Japan; [4]Faculty of Medicine, Department of Microbiology, University of Yamanashi, Yamanashi, Japan; [5]Laboratory of Viral Pathogenesis, Institute for Virus Research, Kyoto University, Kyoto, Japan and [6]AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan
E-mail: tkawa@yamanashi.ac.jp

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Abdool Karim Q, Abdool Karim SS, Frohlich JA *et al.* (2010) Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* 329:1168–74

Ganor Y, Zhou Z, Tudor D *et al.* (2010) Within 1 h, HIV-1 uses viral synapses to enter efficiently the inner, but not outer, foreskin mucosa and engages Langerhans-T cell conjugates. *Mucosal Immunol* 3:506–22

Hu J, Gardner MB, Miller CJ (2000) Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. *J Virol* 74:6087–95

Kawamura T, Cohen SS, Borris DL *et al.* (2000) Candidate microbicides block HIV-1 infection of human immature Langerhans cells within epithelial tissue explants. *J Exp Med* 192: 1491–500

Lederman MM, Offord RE, Hartley O (2006) Microbicides and other topical strategies to prevent vaginal transmission of HIV. *Nat Rev Immunol* 6:371–82

Michailidis E, Marchand B, Kodama EN *et al.* (2009) Mechanism of inhibition of HIV-1 reverse transcriptase by 4′-Ethynyl-2-fluoro-2′-deoxyadenosine triphosphate, a translocation-

defective reverse transcriptase inhibitor. *J Biol Chem* 284:35681–91

Nakata H, Amano M, Koh Y *et al.* (2007) Activity against human immunodeficiency virus type 1, intracellular metabolism, and effects on human DNA polymerases of 4′-ethynyl-2-fluoro-2′-deoxyadenosine. *Antimicrob Agents Chemother* 51:2701–8

Nichols BE, Boucher CA, van de Vijver DA (2011) HIV testing and antiretroviral treatment strategies for prevention of HIV infection: impact on antiretroviral drug resistance. *J Intern Med* 270:532–49

Ogawa Y, Kawamura T, Kimura T *et al.* (2009) Gram-positive bacteria enhance HIV-1 susceptibility in Langerhans cells, but not in dendritic cells, via Toll-like receptor activation. *Blood* 113:5157–66

Ogawa Y, Kawamura T, Matsuzawa T *et al.* (2013) Antimicrobial peptide LL-37 produced by HSV-2-infected keratinocytes enhances HIV infection of Langerhans cells. *Cell Host Microbe* 13:77–86

Ohrui H (2006) 2′-deoxy-4′-C-ethynyl-2-fluoro-adenosine, a nucleoside reverse transcriptase inhibitor, is highly potent against all human immunodeficiency viruses type 1 and has low toxicity. *Chem Rec* 6:133–43

Ohrui H, Kohgo S, Hayakawa H *et al.* (2007) 2′-Deoxy-4′-C-ethynyl-2-fluoroadenosine: a nucleoside reverse transcriptase inhibitor with highly potent activity against wide spectrum of HIV-1 strains, favorable toxic profiles, and stability in plasma. *Nucleosides, Nucleotides Nucleic Acids* 26:1543–6

Rohan LC, Moncla BJ, Kunjara Na Ayudhya RP *et al.* (2010) *In vitro* and *ex vivo* testing of tenofovir shows it is effective as an HIV-1 microbicide. *PLoS One* 5:e9310

Zhou Z, Barry de Longchamps N, Schmitt A *et al.* (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

Zhu T, Mo H, Wang N *et al.* (1993) Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science* 261: 1179–81

Suppression of miR135b Increases the Proliferative Potential of Normal Human Keratinocytes

Journal of Investigative Dermatology (2014) **134,** 1161–1164; doi:10.1038/jid.2013.427; published online 14 November 2013

TO THE EDITOR

Cell fate is regulated by the activation and repression of specific genes (Sonkoly *et al.*, 2007), and microRNAs (miRNAs) are a class of posttranslational regulators of gene expression.

Psoriasis is a hyperproliferative skin disorder (Schneider, 2012) and it has been reported that the stratified epidermis expresses miR135b (Joyce *et al.*, 2011), which suggests that miR135b is closely related to epidermal keratinization. Previously, we isolated three populations of epidermal cells that differed according to their ability to adhere to type IV collagen (Kim *et al.*, 2004). Rapidly adhering (RA) cells, which are considered to be epidermal stem cells, express high levels of α_6 integrin and low levels of CD71. In contrast, slowly adhering (SA) cells express low levels of α_6 integrin and high levels of CD71 (Kim *et al.*, 2004). Type IV collagen-coated dishes were prepared (CellmatrixType IV, Nitta Gelatin, Osaka, Japan) and a subpopulation of cells able to adhere within 10 minutes at 37 °C was selected (RA cells). Thereafter, non-adherent cells were incubated for another 24 hours, and, among them, slowly adhering cells were selected (SA cells). Total RNAs were prepared and real-time reverse-transcriptase–PCR (RT–PCR) analysis was performed. Levels of miR135b were significantly higher in SA cells (three different cell lines from three volunteers, Supplementary Figure S1 online). To test the effects of miR135b, cultured keratinocytes were transfected with miR135b mimic (30 nM, AM17100, PM13044, Ambion, Austin, TX) or mock (as negative controls, 30 nM, AM17110, Ambion). Results showed that miR135b mimic – transfected cells showed abnormal changes compared with mock-transfected cells (Supplementary Figure S2 online). These findings suggested that miR135b initially induced early differentiation of keratinocytes. Therefore, we suppressed miR135b to clarify whether inhibition of miR135b might target and delay differentiation of keratinocytes. The transfection was performed with an anti-miRNA inhibitor (ib-miR135b, AM17000, AM13044, Ambion) designed for hsa-miR135b or a negative control (AM17010, Ambion) at a final concentration of 30 nM according to the manufacturer's instruction. To check transfection efficiency, an FAM-labeled miRNA was transfected and the results showed successful transfection (Supplementary Figure S3 online). Thereafter, the transfected FAM-labeled miRNA was not observed at day 7 after transfection (Supplementary Figure S3 online), which means that the transfected miRNA persisted only for a few days. At every passage, transfection was repeatedly performed, and portions of cells were collected for cell counting, RNA extraction, and protein extraction. RT–PCR analysis showed that transfection of ib-miR135b effectively suppresses miR135b (Figure 1a). Cumulative cell numbers showed a large difference (Figure 1b). A colony assay showed that ib-miR135b-transfected cells showed a higher colony-forming ability than mock-transfected cells (Supplementary Figure S4 online). At the 9th passage, ib-miR135b-transfected cells reached $\sim 5 \times 10^{10}$ cells (Figure 1b). However, mock-transfected cells increased to only 1.6×10^{10} cells (Figure 1b). In addition, mock-transfected cells showed large vacuoles earlier than ib-miR135b-transfected cells (Figure 1b, arrow). Large vacuoles frequently appeared in the cytoplasm, especially in late passages, during the culture of keratinocytes (personal observation). However, these findings are not described in detail in the literature. The vacuolar alteration has

Abbreviations: miRNA, microRNA; RA, rapidly adhering; SA, slowly adhering

Accepted article preview online 15 October 2013; published online 14 November 2013

REVIEW

The Role of Human Dendritic Cells in HIV-1 Infection

Zahra Ahmed[1], Tatsuyoshi Kawamura[2], Shinji Shimada[2] and Vincent Piguet[1]

Dendritic cells (DCs) and their subsets have multifaceted roles in the early stages of HIV-1 transmission and infection. DC studies have led to remarkable discoveries, including identification of restriction factors, cellular structures promoting viral transmission including the infectious synapse or the interplay of the C-type lectins, Langerin on Langerhans cells (LCs), and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin on other DC subsets, limiting or facilitating HIV transmission to $CD4^+$ T cells, respectively. LCs/DCs are also exposed to encountering HIV-1 and other sexually transmitted infections (herpes simplex virus-2, bacteria, fungi), which reprogram HIV-1 interaction with these cells. This review will summarize advances in the role of DCs during HIV-1 infection and discuss their potential involvement in the development of preventive strategies against HIV-1 and other sexually transmitted infections.

Journal of Investigative Dermatology advance online publication, 18 December 2014; doi:10.1038/jid.2014.490

INTRODUCTION

At the start of this century, the Millennium Development Goal 6 called for global efforts to halt and begin to reverse the AIDS epidemic. The UN Political Declaration on HIV/AIDS has supported this and set forth a series of ambitious targets and elimination commitments for 2015. Globally, an estimated 35.3 million people were living with HIV in 2012 (http://www.unaids.org), an increase from previous years as more people are now receiving life-saving anti-retroviral treatment, but significant challenges remain in the fight against HIV. HIV-1 is transmitted through sexual intercourse by crossing epithelial barriers at mucosal surfaces of the genital and anorectal tracts (reviewed in Harman *et al.* (2013b)).

Since their discovery 40 years ago (Steinman and Cohn, 1973), dendritic cells (DCs) and their subsets have been shown to have a major role in immune defence against viral infection by generating and regulating innate and adaptive immune responses. Because of their strategic location at mucosal surfaces, effectiveness at antigen capture, potent migratory ability, and their privileged interaction with effector T cells in lymphoid tissues, DCs are likely critical intermediates of HIV infection and transmission (reviewed in Piguet and Steinman (2007); Haase (2010); Steinman (2012)).

In this review, we will summarize the evidence supporting the importance of DC biology in HIV disease and highlight some of the developments that may have important implications in both prevention and treatment of HIV/AIDS.

BIOLOGY OF DCs AND THEIR SUBSETS

Human skin and mucosal tissues are populated by immature DCs, which are characterized by their specific localization and cell surface receptor expression. DCs capture and internalize, invading pathogens, and subsequently process antigen on major histocompatibility complex class I and class II molecules to $CD8^+$ and $CD4^+$ T cells, respectively (Steinman and Banchereau, 2007). However, antigen presentation alone by DCs is not enough to induce effective T-cell responses against pathogens. $CD4^+$T cells need to differentiate into distinct T helper cell subsets depending on the type of infection into T helper1, T helper2, T helper17, or regulatory T cells. Pathogen recognition is critical to this induction of T-cell differentiation. Despite there being a large variety of pathogens, groups of pathogens share similar structures known as pathogen-associated molecular patterns, which enable their recognition (Akira *et al.*, 2006). DCs express numerous pattern recognition receptors that interact with pathogen-associated molecular patterns inducing cytokine expression and the C-type lectin receptors (CLRs) are a major class of pattern recognition receptors. These CLRs bind pathogens, as well as trigger signaling cascades. The unique position of DCs at the interface with the environment is associated with their pivotal role as sentinels of the immune system. Furthermore, by acting as detectors of foreign danger signals, DCs bridge together the innate and adaptive immune system. Upon pathogen encounter, DCs undergo maturation. During maturation, DCs upregulate molecules on their surface such as the major histocompatibility complex class II, CD80, CD83, CD86, and CD40, which are important for antigen presentation and T-cell stimulation. DCs also migrate from the periphery to the secondary lymphoid organs where they can induce $CD8^+$ and $CD4^+$ T-cell responses (Banchereau and Steinman, 1998).

[1]*Department of Dermatology, Institute of Infection and Immunity, School of Medicine, Cardiff University, University Hospital of Wales, Glamorgan House, Heath Park, Cardiff, UK and* [2]*Faculty of Medicine, Department of Dermatology, University of Yamanashi, Yamanashi, Japan*

Correspondence: Vincent Piguet, Department of Dermatology and Academic Wound Healing, Institute of Infection and Immunity, School of Medicine, Cardiff University, University Hospital of Wales, Glamorgan House, Heath Park, Cardiff CF14 4XN, UK. E-mail: piguetv@cardiff.ac.uk

Abbreviations: APOBEC3G/3F, apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G or 3F; BDCA-1, blood dendritic cell antigen-1; CLRs, C-type lectin receptors; DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; HSV, herpes simplex virus; IS, infectious synapse; LC, Langerhans cell; pDC, plasmacytoid DC; SAMHD1, sterile alpha motif and HD domain 1; SIV, simian immunodeficiency virus

Received 17 March 2014; revised 25 August 2014; accepted 27 September 2014

In human skin, at least three DC subsets have been identified: epidermal $CD207^+$ Langerhans cells (LCs), $CD14^+$ dermal DCs, and $CD14^-CD207^-CD1a^+$ DCs. LCs specifically expressing the CLR CD207 (Langerin) (reviewed in Romani *et al.* (2012); van den Berg and Geijtenbeek (2013)) are mainly found in the epidermal layer (Valladeau *et al.*, 1999, 2000), whereas subsets of dermal DCs, some expressing CD209 dendritic cell–specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), are found throughout the dermis (Lenz *et al.*, 1993; Nestle *et al.*, 1993; Pavli *et al.*, 1993). Langerin expressing cells have also been observed in very low numbers in the human dermis and may represent a subset of the $CD1a^+$ dermal DC population (Harman *et al.*, 2013a) or epidermal LCs transitting through the dermis. In peripheral blood, there are at least two major subsets of DCs, which are both antigen presenting cells with some functional differences: $CD11c^+$ myeloid DCs, which express either CD1c (blood dendritic cell antigen-1 (BDCA-1)) or CD141 (BDCA3), and $CD11c^-CD123^+$ plasmacytoid DCs (pDCs), which express BDCA-2, BDCA-4, and CD123 (interleukin-3 receptor (IL-3R)) and produce a large amount of type I IFN in response to foreign antigen (reviewed in Manches *et al.* (2012); Manches *et al.* (2014)).

In addition, with improvements to both flow cytometric and genomic approaches, several other distinct subsets of DCs have been recently identified. $CLEC9A^+$/$BDCA3^+$ DCs, originally identified in peripheral blood and lymph nodes, have recently been detected in the skin, liver, lung, and intestine. They show a more mature phenotype compared with $CLEC9A^+$/$BDCA3^+$ DCs observed in either blood or lymph nodes, indicating that they may represent a mature stage of differentiation (Chu *et al.*, 2012; Haniffa *et al.*, 2012).

HIV-1 BINDING AND CAPTURE

HIV-1 infection mostly occurs through vaginal or rectal routes, as these submucosal areas are rich in DCs and their subsets. HIV-1 infected DCs are difficult to detect compared with the rapid and massive simian immunodeficiency virus (SIV) infection detected in $CD4^+CCR5^+$ T cells (Brenchley *et al.*, 2004; Mehandru *et al.*, 2004; Li *et al.*, 2005; Mattapallil *et al.*, 2005). Nevertheless, it is likely that DCs, because of their localization, act as early targets for the virus and subsequently contribute to the spread of HIV-1 infection to $CD4^+$ T cells via infectious synapses (IS) (McDonald *et al.*, 2003) and reviewed by Wu and KewalRamani (2006); Piguet and Steinman (2007). Viral uptake mainly occurs via endocytosis after binding to CLRs (DC-SIGN, Langerin, CLEC4A (also known as DC immunoreceptor; Altfeld *et al.*, 2011). See Figure 1.

Apart from HIV-1 binding to DCs expressing the CLR DC-SIGN (Geijtenbeek *et al.*, 2000; Arrighi *et al.*, 2004), other specific DC subsets also express receptors that are able to bind glycoprotein envelope gp120 (Turville *et al.*, 2002; de Witte *et al.*, 2007a). In the subepithelia and lamina propria, DCs bind HIV-1 via DC immunoreceptor (Lambert *et al.*, 2008, 2011). However, gp120 binding and HIV recognition by pDCs are mainly via CD4-mediated endocytosis, despite pDCs also expressing CD4 and the CLR, BDCA-2 (Sandgren *et al.*, 2013; Manches *et al.*, 2014).

HIV-1 capture involving interactions with glycosphingolipids in the virus lipid bilayer have also been described on immature or matured DCs (Gummuluru *et al.*, 2003; Hatch *et al.*, 2009; Izquierdo-Useros *et al.*, 2009).

HIV-1 capture in a glycosphingolipid-dependant manner via the type I IFN-inducible Siglec-1 (CD169) has recently been shown (Puryear *et al.*, 2013). Selective downregulation of CD169 expression or depletion of glycosphingolipids from virions blocked DC-mediated HIV-1 viral capture and trans-infection.

DC lectin receptors are also important molecules involved in foreign antigen presentation (Engering *et al.*, 2002). Most HIV-1 virions captured by DCs are known to be at least in part degraded (Turville *et al.*, 2004; Garcia *et al.*, 2005; Nobile *et al.*, 2005; Blanchet *et al.*, 2010), but HIV-1 binding to DC-SIGN does not lead to full degradation. Instead, some is retained in intracellular compartments connected to the cell surface, often termed virus containing compartment (Garcia *et al.*, 2008; Nikolic *et al.*, 2011), which may facilitate delivery to uninfected T cells via the IS. In macrophages, a similar structure was described (Pelchen-Matthews *et al.*, 2003; Harris *et al.*, 2011). In mature DCs, HIV-1 uptake and infection leads to its co-localization within cholesterol-enriched and tetraspanin-containing compartments, with subsequent delivery of virus via an exosome-like pathway (Izquierdo-Useros *et al.*, 2009). However, in epidermal LCs, virions are directed to acidic compartments for rapid viral degradation (de Witte *et al.*, 2007b) and in pDCs endocytosed HIV-1 localizes to early endosomes triggering type I IFN (O'Brien *et al.*, 2011). This shows that the interplay between HIV-1 and its binding receptors on DC subsets is quite complex as opposing outcomes are seen between C-type lectins on LCs and DCs. This may have implications on designing microbicides, as disarming DC-SIGN on DCs could decrease HIV-1 transmission, whereas disarming Langerin on LCs could prove counterproductive. This could be one reason why Mannan proved ineffective in a SIV model of mucosal transmission, as it inhibited both DC-SIGN and Langerin (Veazey *et al.*, 2005). In contrast, inhibiting CCR5, viral fusion, or both CD4/CCR5 seemed more promising in this model.

TRANS AND *CIS* INFECTIONS OF DC SUBSETS BY HIV-1

Two phases of HIV-1 viral transfer from DCs to T cells have been described (Turville *et al.*, 2004; Wu and KewalRamani, 2006). First, *trans*-infection where the virus is at or near the donor cell surface and transmitted to a different target cell via the IS (McDonald *et al.*, 2003; Arrighi *et al.*, 2004; Nikolic *et al.*, 2011) or via the exosome secretion pathway (Cavrois *et al.*, 2007; Dong *et al.*, 2007; Izquierdo-Useros *et al.*, 2007). This first phase is within 24 hours post HIV-1 exposure and could involve trafficking of captured virus from the endo-lysosomal pathway to the DC-T-cell synapse. In trans-infection, HIV-1 can bind to DC-SIGN and be polarized to a pocket structure connected to the cell surface. After DC-$CD4^+$T cell contact is established, the IS is formed and stabilized through interactions between ICAM-1 and leukocyte function–associated molecule-1 (Garcia *et al.*, 2005;

Human skin DC subsets				Human blood DC subsets (myeloid CD11c$^+$)			Human blood DC subsets (plasmacytoid CD123$^+$)	References
DC subset	LC	CD14$^+$DC	CD1a$^+$DC	BDCA-1 (CD1c)	BDCA3$^-$ (CD141)	BDCA3$^+$	CD123$^+$pDC	(Chu *et al.*, 2012; Fahrbach *et al.*, 2007; Geijtenbeek *et al.*, 2000; Granelli-Piperno *et al.*, 2006; Haniffaa *et al.*, 2012; Liu, 2001; Schmidt *et al.*, 2005; Turville *et al.*, 2002; Wu and KewalRamani, 2006)
Location	Epidermis Gut lumen	Dermis	Dermis	Blood	Blood Secondary lymph organs	Blood	Blood Secondary lymph organs Peripheral tissue (skin, lung, etc.)	
C-type lectin expression (binding to HIV)	Langerin^{++} DCIR DEC205	DCIR DC-SIGN Mannose receptor DEC 205	DCIR Mannose receptor^{++} DEC 205	DCIR DEC205	DEC205 DCIR	CLEC9A	BDCA-2, BDCA-4	
HIV entry receptors and co-receptors involved in binding and internalization by DCs	CD4, cc-chemokine receptor 5 (CCR5) and CXC-chemokine receptor 4 (CXCR4) *Others participating in entry are CCR3, CCR8, CCR9, and CXCR6							*(Altfeld *et al.*, 2011)
Role in HIV infection	Langerin internalizes HIV into degradative Birbeck granules; However HIV can productively infect LCs at higher concentrations and be transmitted to CD4$^+$T cells	Bind to HIV and transmit virus to T cells in draining lymph organs	Bind to HIV and transmit virus to T cells in draining lymph organs	Bind to HIV and transmit virus in both *cis* and *trans* (via DC-SIGN and other receptors) to T cells in the mucosal surfaces and draining lymph organs	Role not yet clear	Role not yet clear	pDCs can be infected productively by HIV-1 Type I IFN production to induce bystander T cell death and inhibit viral replication	(Geijtenbeek *et al.*, 2000; Manches *et al.*, 2012; Schmidt *et al.*, 2005)

Figure 1. Human dendritic cell (DC) subsets and the role in HIV infection. LCs, Langerhans cells; pDCs, plasmacytoid dendritic cells.

Choudhuri *et al.*, 2014). In immature DCs, HIV-1 leads to Cdc42 activation and the formation of a protrusion at the DC cell surface, which facilitates transfer of HIV-1 to CD4$^+$ T cells across the IS (Nikolic *et al.*, 2011). In contrast, in lipopolysaccharide-matured DCs, via actin cytoskeleton rearrangements, the CD4$^+$T cell then extends a filipodium into the pocket on the DC surface to capture HIV-1 (Felts *et al.*, 2010). It is likely that rapid and efficient IS-mediated viral transfer occurs between DCs and CD4$^+$T cells in vitro, but the importance of the IS is more difficult to establish *in vivo*. However, recent data obtained in a humanized mouse model of HIV infection strongly supports a key role for viral cell-to-cell transmission (Murooka *et al.*, 2012). Secondly, *cis*-infection is where HIV-1 can infect target cells and productively replicate, producing progeny virions. These virions then subsequently infect new target cells. This second phase is 24–72 hours after exposure and requires *de novo* replication of virus in DCs.

Long-term HIV transmission mediated by DCs depends on viral production by the DC. It has been observed that immature DCs can retain intact virions for up to 6 days post HIV-1 exposure (Trumpfheller *et al.*, 2003). As most incoming

viruses are degraded in monocyte-derived dendritic cells within 24 hours (Moris *et al.*, 2006), any virus transmitted from DCs to T cells must be a newly synthesized progeny virus.

INTERACTIONS OF HIV WITH LCs

HIV-1 must penetrate the superficial layers of the epidermis to gain access to LCs and resting T cells as potential target cells. The role of LCs in initial HIV/SIV acquisition is supported by *in vivo* and *ex vivo* studies. It has been observed that up to 90% of initially infected target cells were LCs in Rhesus macaques exposed to intra-vaginal SIV (Hu *et al.*, 2000). Human foreskin explants in *ex vivo* experiments show that epidermal LCs are targets for HIV-1 (Ganor *et al.*, 2010; Zhou *et al.*, 2011). LCs may provide protected intracellular transport of HIV to $CD4^+$ T cells in the submucosal lymphoid tissue and then to draining lymph nodes (Kawamura *et al.*, 2000; Cunningham *et al.*, 2008). CD4, CCR5, and CLR expression on LCs in endocervical mucosa and foreskin is thought to be a basis for the preferential sexual transmission of R5 HIV-1. In fact, epidermal LCs are preferentially infected by R5 HIV-1, *ex vivo*, but not X4 HIV-1, and result in high infectivity levels when cocultured with $CD4^+$ T cells (Kawamura *et al.*, 2000; Ogawa *et al.*, 2013). Monocyte-derived LCs are also readily infected in vitro with R5 HIV-1 and treating this cultured LCs with recombinant CD40L can further enhance HIV-1 replication (Kawamura *et al.*, 2001). Although Langerin impairs infection of LCs by HIV-1, there is a saturation of Langerin at higher virus concentrations that overwhelms the protective mechanism of action (de Witte *et al.*, 2007b). Recently, the neuropeptide, calcitonin gene–related peptide, was shown to limit HIV-1 transfer from LCs to T cells through multiple steps, such as increasing Langerin expression (Ganor *et al.*, 2013). It has been reported that vaginal LCs are able to transfer HIV-1 to T cells independent of *cis*-infection (Hladik *et al.*, 2007; Ballweber *et al.*, 2011). Contrary to this, topical application of CCR5 inhibitors has been shown to completely protect Rhesus macaques from intra-vaginal exposure to SHIV, a chimeric simian/human immunodeficiency virus, whereas the CLR-inhibitor, Mannan, could not (Lederman *et al.*, 2004; Veazey *et al.*, 2005). Consistent with these findings, unlike DCs, human LCs have been shown to acquire HIV-1 and transmit the virus to T cells via *cis*-infection rather than *trans*-infection (Kawamura *et al.*, 2003; Peressin *et al.*, 2014).

HIV RESTRICTION AND RECOGNITION

Despite DCs being a vehicle for *trans*-infection, they are themselves poorly infected compared with T cells (Smed-Sorensen *et al.*, 2005).The restriction of X4 HIV-1, and to a lesser degree of R5 HIV-1, replication in DCs could depend on the differential expression of CD4 and chemokine receptors at the cell surface, despite all the receptors being shown to be functionally present (Popov *et al.*, 2005; Pion *et al.*, 2007), and fusion of X4 HIV-1 with immature DCs was seemingly restricted irrespective of surface levels of CXCR4 (Pion *et al.*, 2007). Currently, at least four cellular restriction factors have been shown to block HIV-1 replication at different stages of infection: apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G or 3F (APOBEC3G/3F), tripartite motif-5α, bone marrow stromal cell antigen-2 (tetherin/CD317), and sterile alpha motif and HD domain 1 (SAMHD1; Laguette and Benkirane, 2012) and reviewed in Kirchhoff (2010).

APOBEC3F/G are members of the cytidine deaminase family that can significantly restrict HIV-1 replication in DCs at a post-entry step (Pion *et al.*, 2006; Mohanram *et al.*, 2013). It can be marked for degradation by the virion infectivity factor via ubiquitination and degradation by the proteosome pathway (Sheehy *et al.*, 2003). It was recently shown that A3A is the only member of the APOBEC3 family specifically expressed in primary blood cells of myeloid origins and expression levels increase during HIV-1 spread in infected macrophages (Berger *et al.*, 2011). See Figure 2.

Tripartite motif-5α, important for interspecies retroviral replication blockade, has been recently described as a pattern recognition receptor able to initiate innate immune response upon retroviral capsid lattice recognition, (Battivelli *et al.*, 2011; Pertel *et al.*, 2011). Bone marrow stromal cell antigen-2 is a type I IFN-inducible restriction factor that can potently block HIV-1 release in cell-to-cell transmission of HIV-1. However, it may not be able to restrict DC-mediated HIV-1 capture and transfer (Coleman *et al.*, 2011; Blanchet *et al.*, 2013).

However, the most likely potent restriction in DCs and myeloid cells is the recently identified SAMHD1, blocking at the post-entry step (Hrecka *et al.*, 2011; Laguette *et al.*, 2011). This DC restriction factor was discovered from a study on SIV viral accessory protein X. SAMHD1 functions as a deoxy-nucleoside triphosphate triphosphohydrolase (Goldstone *et al.*, 2011; Lahouassa *et al.*, 2012), which reduces the intracellular dNTP pool, and causes inhibition of reverse transcription and complementary DNA synthesis, thereby depriving HIV-1 of the necessary building blocks. However, this theory was challenged recently, as it was reported that SAMHD1 restriction activity could be separable from its triphosphohydrolase activity (White *et al.*, 2013). Primary myeloid DCs and pDCs express high levels of SAMHD1, resulting in poor induction of SAMHD1 degradation and poor enhancement of HIV-1 infection in these cells (Bloch *et al.*, 2013).

In acute HIV infection, the first detectable plasma cytokines are type I IFNs, which are most likely produced by mucosal and local immune cells (pDCs, DCs, LCs) sensing ssRNA (Stacey *et al.*, 2009).

It is well established that type I IFN is one of the many cytokines that can induce the maturation of DCs (Santini *et al.*, 2000), and it is this maturation that has been shown to block HIV-1 infection at a post-entry level (Dong *et al.*, 2007).

In pDCs, type I IFN production is induced by HIV-1 infection, resulting in the inhibition of HIV-1 replication (Meylan *et al.*, 1993; Groot *et al.*, 2006) independent of SAMHD1 (Goujon *et al.*, 2013). Recently, another molecule, (SLFN 11), induced by type I IFN was recently shown to inhibit the translation of HIV-1 (Li *et al.*, 2012). In addition, a role for IFN-α in restriction was recently demonstrated by showing that HIV-1 uses cleavage and polyadenylation specificity factor subunit 6 and cyclophilins (Nup358 and CypA) to cloak its replication in primary human macrophages

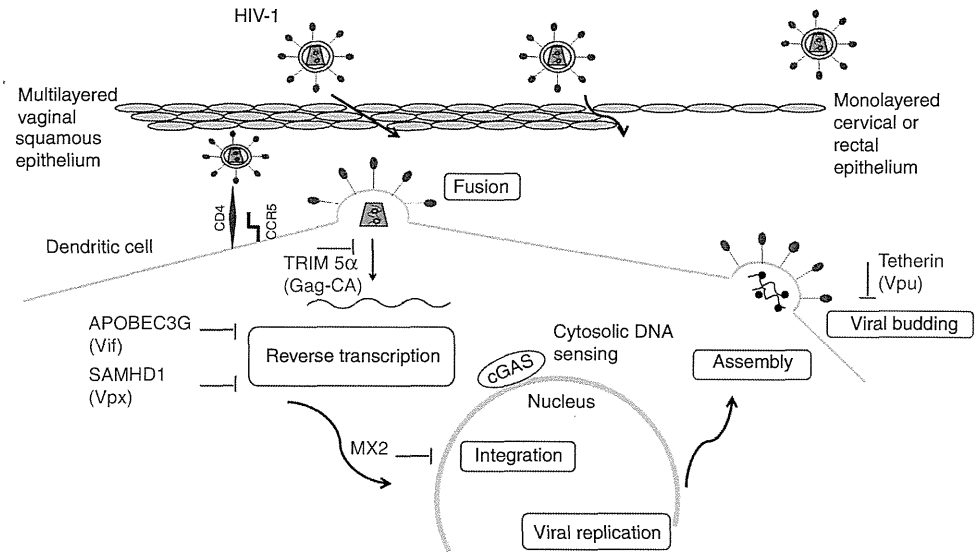


Figure 2. Infection of dendritic cells (DCs) and restriction steps. HIV-1 binds to CD4 and chemokine receptor CCR5, allowing infection of DCs via fusion with cell membrane. However, there are several restriction factors preventing HIV-1 propagation acting at different stages of the viral life cycle shown in blue. Tripartite motif-5α (TRIM-5α) binds to the viral capsid to help disrupt its disassembly. The counteracting viral factor Gag capsid antigen (Gag-CA) is shown in red. Sterile alpha motif and HD domain 1 (SAMHD1) potently blocks reverse transcription at this post-entry step by reducing dNTPs pool, with the counteracting viral factor protein X (Vpx) shown in red. Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) incorporates itself into newly made virions and restricts HIV replication potentially via limiting reverse transctiption and by causing hypermutation. The counteracting viral factor is virion infectivity factor (Vif), shown in red. Myxovirus resistance 2 (MX2) offers IFN-α-mediated resistance to multiple HIV strains by blocking the virus at a late post-entry stage, and finally tetherin prevents viral budding and release by tethering the virus at the cell surface. The counteracting viral factor Vpu is shown in red.

(Manel *et al.*, 2010; Schaller *et al.*, 2011; Rasaiyaah *et al.*, 2013), allowing it to evade innate immune sensors and the induction of innate immune responses.

The IFN-induced myxovirus resistance 2 protein has also been shown to be a key effector of IFN-α-mediated resistance to multiple HIV strains by blocking at a late post-entry stage (Goujon *et al.*, 2013; Kane *et al.*, 2013). Although multiple blocks to infection exist, HIV-1 has been shown to exploit innate immune signaling pathways to facilitate productive infection of DCs. DCs express pattern recognition receptors including Toll-like receptors and CLRs. Toll-like receptor signaling pathways are induced by HIV-1 in DCs to promote replication and cell-to-cell transmission (Gringhuis *et al.*, 2010). Despite HIV-1 replication being very limited in DCs, intracellular recognition of virus and subsequent immune activation appears to be subdued by host factors (Iwasaki, 2012). Deletion of the three prime repair exonuclease leads to accumulation of defective HIV-1 viral DNA products and production of type I IFN (Stetson *et al.*, 2008). This has been shown to be via activation of an unknown cytoplasmic sensor, mediated by stimulator of IFN genes, TANK-binding kinase 1, and IFN regulatory factor 3 (Yan *et al.*, 2010). The recently identified enzyme cGAMP synthase has been shown as a cytosolic DNA sensor that triggers the production of type I IFNs and other cytokines (Lahaye *et al.*, 2013; Sun *et al.*, 2013). It functions as a second messenger binding to and activating the endoplasmic reticulum protein stimulator of IFN genes (Ablasser *et al.*, 2013; Zhang *et al.*, 2013; Gao *et al.*, 2013b). Stimulator of IFN genes subsequently activates TANK-binding kinase 1, which then activates transcription factors NF-κB and IFN regulatory factor 3 to induce IFNs and other cytokines (Barber, 2011).

HIV-1 can induce the production of cGAMP via cGAMP synthase under permissive conditions, suggesting that cGAMP may also be employed to bypass the block of innate immune responses against HIV (Gao *et al.*, 2013a).

Together, these recent findings indicate that there are yet undiscovered additional restriction factors relating to type I IFN signaling in DCs and that these have the potential to be exploited to fight retroviral infections.

EFFECT OF COINFECTIONS ON LCs AND HIV-1 SUSCEPTIBILITY

The risk of HIV-1 infection increases in the presence of coinfection from sexually transmitted diseases by increasing inflammation and influx of activated $CD4^+$ T cells (de Jong *et al.*, 2008). Any protective function of Langerin against HIV-1 is also removed by sexually transmitted disease coinfections such as herpes simplex virus (HSV) or Candida species (de Jong *et al.*, 2010a). Both HSV-2 and Candida species occupy the Langerin receptor, obstructing Langerin function and subsequently increasing the risk of HIV-1 infection (Turville *et al.*, 2003; de Jong *et al.*, 2010b). HSV-2 is also able to infect LCs, decreasing Langerin expression and its protective role. HSV-2 enhances the HIV susceptibility of LCs within skin explants. Epidermal LCs exposed to HSV-2 and HIV-1 were rarely co-infected with these viruses. Instead, keratinocytes infected with HSV-2 increased their production of the anti-microbial peptide LL-37, which enhanced HIV susceptibility in monocyte-derived LC through upregulating their expression

of HIV-1 co-receptors, but not affecting restriction factors such as Langerin, APOBEC3G, or SAMHD1 (Ogawa *et al.*, 2013). Sexually transmitted pathogens such as *Candida albicans* and *Neisseria gonorrhea* and pathogenic bacteria from bacterial vaginosis such as *Listeria monocytogenes* and *Staphylococcus aureus* have been shown to improve HIV-1 capture and/or infection in LCs, which led to increased transmission to T cells via Toll-like receptors2 triggering, and subsequently reduced expression of Langerin and APOBEC3G (Ogawa *et al.*, 2009; de Jong *et al.*, 2010a). Moreover, *C.albicans, N.gonorrhea*, and HSV-2 enhance HIV-1 production in LCs and transfer to T cells via tumor necrosis factor-α local production (de Jong *et al.*, 2008, 2010a). Therefore, it appears that during coinfections, concomitant sexually transmitted diseases infection alters HIV-1 susceptibility and the functional ability of Langerin in LCs directly and indirectly, thereby potentially increasing the risk of acquiring HIV-1 infection and transmission of HIV-1 to T cells.

CONCLUSION AND FUTURE DIRECTIONS

Of the vaccines tested in clinical efficacy trials in humans over the last 30 years, the RV144 phase 3 study conducted in a low-risk population in Thailand showed a 31% reduction in the rate of HIV-1 acquisition. (Rerks-Ngarm *et al.*, 2009). Antibodies elicited by the vaccine against the HIV-1 envelope correlated with a reduced risk of infection (Haynes *et al.*, 2012; Pollara *et al.*, 2014). DCs are likely to be important targets for any vaccine candidate, because of their critical role in the initiation and shaping of adaptive immune responses. Despite the above mentioned advances in vaccine development and in the interim absence of an effective vaccine, chemoprophylaxis in the form of topical microbicides may be an alternative. In a double-blind, randomized controlled trial on the effectiveness of a 1% vaginal gel formulation, Tenofovir (reverse transcriptase inhibitor) showed up to 39% reduction in HIV-1 incidence in women (Abdool Karim *et al.*, 2010; Cottrell and Kashuba, 2014).

In another study, it was demonstrated that oral CCR5 inhibitor, Maraviroc, can block HIV-1 infection of epidermal LCs in an *ex vivo* model and a newly discovered reverse transcriptase inhibitor, EFdA, can be a potent microbicide to prevent HIV-1 infection instead of condom use (Matsuzawa *et al.*, 2013, 2014)

A further development is an experimental injectable pre-exposure prophylaxis drug GSK 744 (HIV-1 integrase inhibitor) shown in macaques to give long lasting protection against a hybrid simian-human AIDS virus (Andrews *et al.*, 2014).

In addition, stimulation of autophagy in DCs leads to more rapid and robust adaptive immune responses against HIV-1 (Blanchet *et al.*, 2010), therefore targeting stimulation of autophagy in the early events of HIV-1 infection in mucosal tissues could represent a method to circumvent viral propagation (Shoji-Kawata *et al.*, 2013).

In summary, future studies will need to delineate which human DC subset(s) is central to antigen uptake and induction of immunity, in order to enhance microbicides and vaccine efficacy in preventing HIV-1 transmission across mucosal surfaces.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by Wellcome Trust Institutional Strategic Support Fund to Cardiff University (506398) and Gates Foundation Grant to VP; ZA is a recipient of WCAT fellowship (http://www.walesdeanery.org/index.php/wcat.html). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

Abdool Karim Q, Abdool Karim SS, Frohlich JA *et al.* (2010) Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* 329:1168–74

Ablasser A, Goldeck M, Cavlar T *et al.* (2013) cGAS produces a 2′-5′-linked cyclic dinucleotide second messenger that activates STING. *Nature* 498:380–4

Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. *Cell* 124:783–801

Altfeld M, Fadda L, Frleta D *et al.* (2011) DCs and NK cells: critical effectors in the immune response to HIV-1. *Nat Rev Immunol* 11:176–86

Andrews CD, Spreen WR, Mohri H *et al.* (2014) Long-acting integrase inhibitor protects macaques from intrarectal simian/human immunodeficiency virus. *Science* 343:1151–4

Arrighi JF, Pion M, Garcia E *et al.* (2004) DC-SIGN-mediated infectious synapse formation enhances X4 HIV-1 transmission from dendritic cells to T cells. *J Exp Med* 200:1279–88

Ballweber L, Robinson B, Kreger A *et al.* (2011) Vaginal langerhans cells nonproductively transporting HIV-1 mediate infection of T cells. *J Virol* 85:13443–7

Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. *Nature* 392:245–52

Barber GN (2011) Cytoplasmic DNA innate immune pathways. *Immunol Rev* 243:99–108

Battivelli E, Migraine J, Lecossier D *et al.* (2011) Modulation of TRIM5alpha activity in human cells by alternatively spliced TRIM5 isoforms. *J Virol* 85:7828–35

Berger G, Durand S, Fargier G *et al.* (2011) APOBEC3A is a specific inhibitor of the early phases of HIV-1 infection in myeloid cells. *PLoS Pathog* 7:e1002221

Blanchet FP, Moris A, Nikolic DS *et al.* (2010) Human immunodeficiency virus-1 inhibition of immunoamphisomes in dendritic cells impairs early innate and adaptive immune responses. *Immunity* 32:654–69

Blanchet FP, Stalder R, Czubala M *et al.* (2013) TLR-4 engagement of dendritic cells confers a BST- 2/tetherin-mediated restriction of HIV-1 infection to CD4+ T cells across the virological synapse. *Retrovirology* 10:6

Bloch N, O'Brien M, Norton TD *et al.* (2013) HIV Type 1 infection of plasmacytoid and myeloid dendritic cells is restricted by high levels of SAMHD1 and cannot be counteracted by Vpx. *AIDS Res Hum Retroviruses* 30:195–203

Brenchley JM, Schacker TW, Ruff LE *et al.* (2004) CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* 200:749–59

Cavrois M, Neidleman J, Kreisberg JF *et al.* (2007) In vitro derived dendritic cells trans-infect CD4 T cells primarily with surface-bound HIV-1 virions. *PLoS Pathog* 3:e4

Choudhuri K, Llodra J, Roth EW *et al.* (2014) Polarized release of T-cell-receptor-enriched microvesicles at the immunological synapse. *Nature* 507:118–23

Chu CC, Ali N, Karagiannis P *et al.* (2012) Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. *J Exp Med* 209:935–45

Coleman CM, Spearman P, Wu L (2011) Tetherin does not significantly restrict dendritic cell- mediated HIV-1 transmission and its expression is upregulated by newly synthesized HIV-1 Nef. *Retrovirology* 8:26

Cottrell ML, Kashuba AD (2014) Topical microbicides and HIV prevention in the female genital tract. *J Clin Pharmacol* 54:603–15

Cunningham AL, Carbone F, Geijtenbeek TB (2008) Langerhans cells and viral immunity. *Eur J Immunol* 38:2377–85

de Jong MA, de Witte L, Oudhoff MJ *et al.* (2008) TNF-alpha and TLR agonists increase susceptibility to HIV-1 transmission by human Langerhans cells *ex vivo*. *J Clin Invest* 118:3440–52

de Jong MA, de Witte L, Taylor ME *et al.* (2010a) Herpes simplex virus type 2 enhances HIV-1 susceptibility by affecting Langerhans cell function. *J Immunol* 185:1633–41

de Jong MA, Vriend LE, Theelen B *et al.* (2010b) C-type lectin Langerin is a beta-glucan receptor on human Langerhans cells that recognizes opportunistic and pathogenic fungi. *Mol Immunol* 47:1216–25

de Witte L, Bobardt M, Chatterji U *et al.* (2007a) Syndecan-3 is a dendritic cell-specific attachment receptor for HIV-1. *Proc Natl Acad Sci USA* 104:19464–9

de Witte L, Nabatov A, Pion M *et al.* (2007b) Langerin is a natural barrier to HIV-1 transmission by Langerhans cells. *Nat Med* 13:367–71

Dong C, Janas AM, Wang JH *et al.* (2007) Characterization of human immunodeficiency virus type 1 replication in immature and mature dendritic cells reveals dissociable cis- and trans-infection. *J Virol* 81:11352–62

Engering A, Geijtenbeek TB, van Vliet SJ *et al.* (2002) The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J Immunol* 168:2118–26

Fahrbach KM, Barry SM, Ayehunie S *et al.* (2007) Activated CD34-derived Langerhans cells mediate transinfection with human immunodeficiency virus. *J Virol* 81:6858–68

Felts RL, Narayan K, Estes JD *et al.* (2010) 3D visualization of HIV transfer at the virological synapse between dendritic cells and T cells. *Proc Natl Acad Sci USA* 107:13336–41

Ganor Y, Drillet-Dangeard AS, Lopalco L *et al.* (2013) Calcitonin gene-related peptide inhibits Langerhans cell-mediated HIV-1 transmission. *J Exp Med* 210:2161–70

Ganor Y, Zhou Z, Tudor D *et al.* (2010) Within 1h, HIV-1 uses viral synapses to enter efficiently the inner, but not outer, foreskin mucosa and engages Langerhans-T cell conjugates. *Mucosal Immunol* 3:506–22

Gao D, Wu J, Wu YT *et al.* (2013a) Cyclic GMP-AMP synthase is an innate immune sensor of HIV and other retroviruses. *Science* 341:903–6

Gao P, Ascano M, Wu Y *et al.* (2013b) Cyclic [G(2',5')pA(3',5')p] is the metazoan second messenger produced by DNA-activated cyclic GMP-AMP synthase. *Cell* 153:1094–107

Garcia E, Nikolic DS, Piguet V (2008) HIV-1 replication in dendritic cells occurs through a tetraspanin- containing compartment enriched in AP-3. *Traffic* 9:200–14

Garcia E, Pion M, Pelchen-Matthews A *et al.* (2005) HIV-1 trafficking to the dendritic cell-T-cell infectious synapse uses a pathway of tetraspanin sorting to the immunological synapse. *Traffic* 6:488–501

Geijtenbeek TB, Kwon DS, Torensma R *et al.* (2000) DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* 100:587–97

Goldstone DC, Ennis-Adeniran V, Hedden JJ *et al.* (2011) HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase. *Nature* 480:379–82

Goujon C, Moncorge O, Bauby H *et al.* (2013) Human MX2 is an interferon-induced post-entry inhibitor of HIV-1 infection. *Nature* 502:559–62

Granelli-Piperno A, Shimeliovich I, Pack M *et al.* (2006) HIV-1 selectively infects a subset of nonmaturing BDCA1-positive dendritic cells in human blood. *J Immunol* 176:991–8

Gringhuis SI, van der Vlist M, van den Berg LM *et al.* (2010) HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells. *Nat Immunol* 11:419–26

Groot F, van Capel TM, Kapsenberg ML *et al.* (2006) Opposing roles of blood myeloid and plasmacytoid dendritic cells in HIV-1 infection of T cells: transmission facilitation versus replication inhibition. *Blood* 108:1957–64

Gummuluru S, Rogel M, Stamatatos L *et al.* (2003) Binding of human immunodeficiency virus type 1 to immature dendritic cells can occur independently of DC-SIGN and mannose binding C-type lectin receptors via a cholesterol-dependent pathway. *J Virol* 77:12865–74

Haase AT (2010) Targeting early infection to prevent HIV-1 mucosal transmission. *Nature* 464:217–23

Haniffa M, Shin A, Bigley V *et al.* (2012) Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. *Immunity* 37:60–73

Harman AN, Bye CR, Nasr N *et al.* (2013a) Identification of lineage relationships and novel markers of blood and skin human dendritic cells. *J Immunol* 190:66–79

Harman AN, Kim M, Nasr N *et al.* (2013b) Tissue dendritic cells as portals for HIV entry. *Rev Med Virol* 23:319–33

Harris A, Borgnia MJ, Shi D *et al.* (2011) Trimeric HIV-1 glycoprotein gp140 immunogens and native HIV-1 envelope glycoproteins display the same closed and open quaternary molecular architectures. *Proc Natl Acad Sci USA* 108:11440–5

Hatch SC, Archer J, Gummuluru S (2009) Glycosphingolipid composition of human immunodeficiency virus type 1 (HIV-1) particles is a crucial determinant for dendritic cell-mediated HIV-1 trans- infection. *J Virol* 83:3496–506

Haynes BF, Gilbert PB, McElrath MJ *et al.* (2012) Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med* 366:1275–86

Hladik F, Sakchalathorn P, Ballweber L *et al.* (2007) Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1. *Immunity* 26:257–70

Hrecka K, Hao C, Gierszewska M *et al.* (2011) Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. *Nature* 474:658–61

Hu J, Gardner MB, Miller CJ (2000) Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. *J Virol* 74:6087–95

Iwasaki A (2012) Innate immune recognition of HIV-1. *Immunity* 37:389–98

Izquierdo-Useros N, Blanco J, Erkizia I *et al.* (2007) Maturation of blood-derived dendritic cells enhances human immunodeficiency virus type 1 capture and transmission. *J Virol* 81:7559–70

Izquierdo-Useros N, Naranjo-Gomez M, Archer J *et al.* (2009) Capture and transfer of HIV-1 particles by mature dendritic cells converges with the exosome-dissemination pathway. *Blood* 113:2732–41

Kane M, Yadav SS, Bitzegeio J *et al.* (2013) MX2 is an interferon-induced inhibitor of HIV-1 infection. *Nature* 502:563–6

Kawamura T, Cohen SS, Borris DL *et al.* (2000) Candidate microbicides block HIV-1 infection of human immature Langerhans cells within epithelial tissue explants. *J Exp Med* 192:1491–500

Kawamura T, Gulden FO, Sugaya M *et al.* (2003) R5 HIV productively infects Langerhans cells, and infection levels are regulated by compound CCR5 polymorphisms. *Proc Natl Acad Sci USA* 100:8401–6

Kawamura T, Qualbani M, Thomas EK *et al.* (2001) Low levels of productive HIV infection in Langerhans cell-like dendritic cells differentiated in the presence of TGF-beta1 and increased viral replication with CD40 ligand-induced maturation. *Eur J Immunol* 31:360–8

Kirchhoff F (2010) Immune evasion and counteraction of restriction factors by HIV-1 and other primate lentiviruses. *Cell Host Microbe* 8:55–67

Laguette N, Benkirane M (2012) How SAMHD1 changes our view of viral restriction. *Trends Immunol* 33:26–33

Laguette N, Sobhian B, Casartelli N *et al.* (2011) SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature* 474:654–7

Lahaye X, Satoh T, Gentili M *et al.* (2013) The capsids of HIV-1 and HIV-2 determine immune detection of the viral cDNA by the innate sensor cGAS in dendritic cells. *Immunity* 39:1132–42

Lahouassa H, Daddacha W, Hofmann H *et al.* (2012) SAMHD1 restricts the replication of human immunodeficiency virus type 1 by depleting the intracellular pool of deoxynucleoside triphosphates. *Nat Immunol* 13:223–8

Lambert AA, Barabe F, Gilbert C *et al.* (2011) DCIR-mediated enhancement of HIV-1 infection requires the ITIM-associated signal transduction pathway. *Blood* 117:6589–99

Lambert AA, Gilbert C, Richard M *et al.* (2008) The C-type lectin surface receptor DCIR acts as a new attachment factor for HIV-1 in dendritic cells and contributes to trans- and cis-infection pathways. *Blood* 112:1299–307

Lederman MM, Veazey RS, Offord R *et al.* (2004) Prevention of vaginal SHIV transmission in rhesus macaques through inhibition of CCR5. *Science* 306:485–7

Lenz A, Heine M, Schuler G *et al.* (1993) Human and murine dermis contain dendritic cells. Isolation by means of a novel method and phenotypical and functional characterization. *J Clin Invest* 92:2587–96

Li M, Kao E, Gao X *et al.* (2012) Codon-usage-based inhibition of HIV protein synthesis by human schlafen 11. *Nature* 491:125–8

Li Q, Duan L, Estes JD *et al.* (2005) Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* 434:1148–52

Liu YJ (2001) Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* 106:259–62

Manches O, Fernandez MV, Plumas J *et al.* (2012) Activation of the noncanonical NF-kappaB pathway by HIV controls a dendritic cell immunoregulatory phenotype. *Proc Natl Acad Sci USA* 109:14122–7

Manches O, Frleta D, Bhardwaj N (2014) Dendritic cells in progression and pathology of HIV infection. *Trends Immunol* 35:114–22

Manel N, Hogstad B, Wang Y *et al.* (2010) A cryptic sensor for HIV-1 activates antiviral innate immunity in dendritic cells. *Nature* 467:214–7

Matsuzawa T, Kawamura T, Ogawa Y *et al.* (2014) EFdA, a reverse transcriptase inhibitor, potently blocks HIV-1 *ex vivo* infection of Langerhans cells within epithelium. *J Invest Dermatol* 134:1158–61

Matsuzawa T, Kawamura T, Ogawa Y *et al.* (2013) Oral administration of the CCR5 inhibitor, maraviroc, blocks HIV *ex vivo* infection of Langerhans cells within the epithelium. *J Invest Dermatol* 133:2803–5

Mattapallil JJ, Douek DC, Hill B *et al.* (2005) Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* 434:1093–7

McDonald D, Wu L, Bohks SM *et al.* (2003) Recruitment of HIV and its receptors to dendritic cell-T cell junctions. *Science* 300:1295–7

Mehandru S, Poles MA, Tenner-Racz K *et al.* (2004) Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* 200:761–70

Meylan PR, Guatelli JC, Munis JR *et al.* (1993) Mechanisms for the inhibition of HIV replication by interferons-alpha, -beta, and -gamma in primary human macrophages. *Virology* 193:138–48

Mohanram V, Skold AE, Bachle SM *et al.* (2013) IFN-alpha induces APOBEC3G, F, and A in immature dendritic cells and limits HIV-1 spread to CD4+ T cells. *J Immunol* 190:3346–53

Moris A, Pajot A, Blanchet F *et al.* (2006) Dendritic cells and HIV-specific CD4+ T cells: HIV antigen presentation, T-cell activation, and viral transfer. *Blood* 108:1643–51

Murooka TT, Deruaz M, Marangoni F *et al.* (2012) HIV-infected T cells are migratory vehicles for viral dissemination. *Nature* 490:283–7

Nestle FO, Zheng XG, Thompson CB *et al.* (1993) Characterization of dermal dendritic cells obtained from normal human skin reveals phenotypic and functionally distinctive subsets. *J Immunol* 151:6535–45

Nikolic DS, Lehmann M, Felts R *et al.* (2011) HIV-1 activates Cdc42 and induces membrane extensions in immature dendritic cells to facilitate cell-to-cell virus propagation. *Blood* 118:4841–52

Nobile C, Petit C, Moris A *et al.* (2005) Covert human immunodeficiency virus replication in dendritic cells and in DC-SIGN-expressing cells promotes long-term transmission to lymphocytes. *J Virol* 79:5386–99

O'Brien M, Manches O, Sabado RL *et al.* (2011) Spatiotemporal trafficking of HIV in human plasmacytoid dendritic cells defines a persistently IFN-alpha-producing and partially matured phenotype. *J Clin Invest* 121:1088–101

Ogawa Y, Kawamura T, Kimura T *et al.* (2009) Gram-positive bacteria enhance HIV-1 susceptibility in Langerhans cells, but not in dendritic cells, via Toll-like receptor activation. *Blood* 113:5157–66

Ogawa Y, Kawamura T, Matsuzawa T *et al.* (2013) Antimicrobial peptide LL-37 produced by HSV-2- infected keratinocytes enhances HIV infection of Langerhans cells. *Cell Host Microbe* 13:77–86

Pavli P, Hume DA, Van De Pol E *et al.* (1993) Dendritic cells, the major antigen-presenting cells of the human colonic lamina propria. *Immunology* 78:132–41

Pelchen-Matthews A, Kramer B, Marsh M (2003) Infectious HIV-1 assembles in late endosomes in primary macrophages. *J Cell Biol* 162:443–55

Peressin M, Proust A, Schmidt S *et al.* (2014) Efficient transfer of HIV-1 in trans and in cis from Langerhans dendritic cells and macrophages to autologous T lymphocytes. *AIDS* 28:667–77

Pertel T, Hausmann S, Morger D *et al.* (2011) TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature* 472:361–5

Piguet V, Steinman RM (2007) The interaction of HIV with dendritic cells: outcomes and pathways. *Trends Immunol* 28:503–10

Pion M, Arrighi JF, Jiang J *et al.* (2007) Analysis of HIV-1-X4 fusion with immature dendritic cells identifies a specific restriction that is independent of CXCR4 levels. *J Invest Dermatol* 127:319–23

Pion M, Granelli-Piperno A, Mangeat B *et al.* (2006) APOBEC3G/3F mediates intrinsic resistance of monocyte-derived dendritic cells to HIV-1 infection. *J Exp Med* 203:2887–93

Pollara J, Bonsignori M, Moody MA *et al.* (2014) HIV-1 vaccine-induced C1 and V2 Env-specific antibodies synergize for increased antiviral activities. *J Virol* 88:7715–26

Popov S, Chenine AL, Gruber A *et al.* (2005) Long-term productive human immunodeficiency virus infection of CD1a-sorted myeloid dendritic cells. *J Virol* 79:602–8

Puryear WB, Akiyama H, Geer SD *et al.* (2013) Interferon-inducible mechanism of dendritic cell- mediated HIV-1 dissemination is dependent on Siglec-1/CD169. *PLoS Pathog* 9:e1003291

Rasaiyaah J, Tan CP, Fletcher AJ *et al.* (2013) HIV-1 evades innate immune recognition through specific cofactor recruitment. *Nature* 503:402–5

Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S *et al.* (2009) Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* 361:2209–20

Romani N, Brunner PM, Stingl G (2012) Changing views of the role of Langerhans cells. *J Invest Dermatol* 132:872–81

Sandgren KJ, Smed-Sorensen A, Forsell MN *et al.* (2013) Human plasmacytoid dendritic cells efficiently capture HIV-1 envelope glycoproteins via CD4 for antigen presentation. *J Immunol* 191:60–9

Santini SM, Lapenta C, Logozzi M *et al.* (2000) Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. *J Exp Med* 191:1777–88

Schaller T, Ocwieja KE, Rasaiyaah J *et al.* (2011) HIV-1 capsid-cyclophilin interactions determine nuclear import pathway, integration targeting and replication efficiency. *PLoS Pathog* 7:e1002439

Schmidt B, Ashlock BM, Foster H *et al.* (2005) HIV-infected cells are major inducers of plasmacytoid dendritic cell interferon production, maturation, and migration. *Virology* 343:256–66

Sheehy AM, Gaddis NC, Malim MH (2003) The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nat Med* 9:1404–7

Shoji-Kawata S, Sumpter R, Leveno M *et al.* (2013) Identification of a candidate therapeutic autophagy-inducing peptide. *Nature* 494:201–6

Smed-Sorensen A, Lore K, Vasudevan J *et al.* (2005) Differential susceptibility to human immunodeficiency virus type 1 infection of myeloid and plasmacytoid dendritic cells. *J Virol* 79:8861–9

Stacey AR, Norris PJ, Qin L *et al.* (2009) Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more modest and delayed responses in acute hepatitis B and C virus infections. *J Virol* 83:3719–33

Steinman RM (2012) Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol* 30:1–22

Steinman RM, Banchereau J (2007) Taking dendritic cells into medicine. *Nature* 449:419–26

Steinman RM, Cohn ZA (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137:1142–62

Stetson DB, Ko JS, Heidmann T *et al.* (2008) Trex1 prevents cell-intrinsic initiation of autoimmunity. *Cell* 134:587–98

Sun L, Wu J, Du F *et al.* (2013) Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* 339:786–91

Trumpfheller C, Park CG, Finke J *et al.* (2003) Cell type-dependent retention and transmission of HIV-1 by DC-SIGN. *Int Immunol* 15:289–98

Turville S, Wilkinson J, Cameron P *et al.* (2003) The role of dendritic cell C-type lectin receptors in HIV pathogenesis. *J Leukoc Biol* 74:710–8

Turville SG, Cameron PU, Handley A *et al.* (2002) Diversity of receptors binding HIV on dendritic cell subsets. *Nat Immunol* 3:975–83

Turville SG, Santos JJ, Frank I *et al.* (2004) Immunodeficiency virus uptake, turnover, and 2-phase transfer in human dendritic cells. *Blood* 103:2170–9

Valladeau J, Duvert-Frances V, Pin JJ *et al.* (1999) The monoclonal antibody DCGM4 recognizes Langerin, a protein specific of Langerhans cells, and is rapidly internalized from the cell surface. *Eur J Immunol* 29:2695–704

Valladeau J, Ravel O, Dezutter-Dambuyant C *et al.* (2000) Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* 12:71–81

van den Berg LM, Geijtenbeek TB (2013) Antiviral immune responses by human langerhans cells and dendritic cells in HIV-1 infection. *Adv Exp Med Biol* 762:45–70

Veazey RS, Klasse PJ, Schader SM *et al.* (2005) Protection of macaques from vaginal SHIV challenge by vaginally delivered inhibitors of virus-cell fusion. *Nature* 438:99–102

White TE, Brandariz-Nunez A, Valle-Casuso JC *et al.* (2013) The retroviral restriction ability of SAMHD1, but not its deoxynucleotide triphosphohydrolase activity, is regulated by phosphorylation. *Cell Host Microbe* 13:441–51

Wu L, KewalRamani VN (2006) Dendritic-cell interactions with HIV: infection and viral dissemination. *Nat Rev Immunol* 6:859–68

Yan N, Regalado-Magdos AD, Stiggelbout B *et al.* (2010) The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1. *Nat Immunol* 11:1005–13

Zhang X, Shi H, Wu J *et al.* (2013) Cyclic GMP-AMP containing mixed phosphodiester linkages is an endogenous high-affinity ligand for STING. *Mol Cell* 51:226–35

Zhou Z, Barry de Longchamps N, Schmitt A *et al.* (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

Antiviral Therapy 2014; 19:179–189 (doi: 10.3851/IMP2697)

Original article

Delayed emergence of HIV-1 variants resistant to 4′-ethynyl-2-fluoro-2′-deoxyadenosine: comparative sequential passage study with lamivudine, tenofovir, emtricitabine and BMS-986001

Kenji Maeda[1], Darshan V Desai[1], Manabu Aoki[2,3], Hirotomo Nakata[1,3], Eiichi N Kodama[4], Hiroaki Mitsuya[1,3]*

[1]Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

[2]Department of Medical Technology, Kumamoto Health Science University, Kumamoto, Japan

[3]Departments of Hematology and Infectious Diseases, Kumamoto University Graduate School of Medical and Pharmaceutical Sciences, Kumamoto, Japan

[4]Division of Emerging Infectious Diseases, Tohoku University Hospital, Tohoku Medical Megabank Organization, Sendai, Japan

*Corresponding author e-mail: maedak@mail.nih.gov

Background: 4′-Ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) contains an ethynyl moiety and the 3′-hydroxyl and exerts highly potent activity against various HIV type-1 (HIV-1) strains including multi-drug-resistant variants.

Methods: Comparative selection passages against EFdA, lamivudine (3TC), tenofovir disoproxil fumarate (TDF), emtricitabine (FTC) or BMS-986001 (Ed4T) were conducted using a mixture of 11 highly multi-drug-resistant clinical HIV-1 isolates (HIV_{11MIX}) as a starting virus population.

Results: Before selection, HIV_{11MIX} was sensitive to EFdA with a 50% inhibitory concentration (IC_{50}) of 0.032 μM, less susceptible to TDF and Ed4T with IC_{50}s of 0.57 and 2.6 μM, respectively, and highly resistant to 3TC and FTC with IC_{50}s>10 μM. IC_{50}s of TDF against HIV_{11MIX} exposed to EFdA and TDF for 17 ($HIV_{11MIX}^{EFdA-P17}$) and 14 ($HIV_{11MIX}^{TDF-P14}$) passages were 8 and >10 μM, respectively, while EFdA remained active against $HIV_{11MIX}^{EFdA-P17}$ and $HIV_{11MIX}^{TDF-P14}$ with IC_{50}s of 0.15 and 0.1 μM, respectively. Both selected variants were highly resistant against zidovudine, 3TC, Ed4T and FTC (IC_{50} values >10 μM).

Conclusions: The present data demonstrate that HIV_{11MIX} developed resistance more rapidly against 3TC, FTC, TDF and Ed4T than against EFdA and that EFdA remained substantially active against TDF- and EFdA-selected variants. Thus, EFdA has a favourable resistance profile and represents a potentially promising new-generation nucleoside reverse transcriptase inhibitor.

Introduction

Since the development of zidovudine (AZT), the first antiretroviral agent against AIDS [1], a number of therapeutics have been added to the armamentarium in the fight against HIV type-1 (HIV-1) infection [2,3]. The combination antiretroviral therapy (cART) using such agents has been shown to potently suppress HIV-1 replication and extend the life expectancy of HIV-1-infected individuals [4]. However, we have encountered a number of challenges in achieving the optimal benefits of the currently available therapeutics in individuals receiving cART. They include: drug-related toxicities; only partial restoration of immunologic functions achieved once individuals developed AIDS; development of various cancers as a consequence of survival prolongation; flare-up of inflammation in individuals receiving cART or immune reconstitution syndrome; and increased cost of antiviral therapy [5,6]. Importantly, HIV-1 is believed to ultimately develop resistance to any existing antiretroviral regimens. It is thus crucial that efforts are continued to develop more potent and safer novel therapeutics, which are active against wild-type isolates and existing drug-resistant HIV-1 variants, and which delay or prevent the emergence of HIV-1 variants resistant to such novel therapeutics [3,7,8].

Currently, one of the most commonly used NRTIs is tenofovir disoproxil fumarate (TDF), which is a prodrug form of tenofovir. TDF has mostly been used as a once-a-day fixed-dose tablet (Truvada®) combined

with emtricitabine (FTC) for treating HIV-1-infected individuals including those who carry HIV-1 strains that are resistant to other existing nucleoside reverse transcriptase inhibitors (NRTIs) but has also been used for treating patients with HBV infection. TDF showed no significant side effects in the initial clinical trials; however, severe acute renal failure was seen in certain patients receiving TDF [9,10]. Moreover, there is a substantial concern that TDF causes nephrotoxicity and osteoporosis with the long-term administration, particularly in aged individuals with HIV-1 infection [9,11]. In addition, K65R substitution in reverse transcriptase (RT), identified in clinical HIV-1 strains isolated from those receiving TDF is known to reduce the antiretroviral activity of TDF [12]. Although the K65R substitution is not often selected with TDF in combination with other drugs [13], the long-term use of TDF has resulted in the increased occurrence of the substitution. Thus, the discovery of the next generation of RT inhibitors, which have less toxicity and do not possess cross-resistance with TDF is needed as a component of salvage therapy for patients, who do not tolerate TDF or have acquired TDF-resistant HIV-1 strains.

All currently approved NRTIs lack a 3′-OH moiety and it had been thought to be essential for NRTIs to possess the activity as chain terminators. However, we have reported that a number of 4′-ethynyl-2′-deoxynucleoside analogues (EdNs), which maintain the 3′-OH in their sugar moiety, have activity against HIV-1 [14–16]. Through the optimization efforts of such EdNs, we found that 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA; Figure 1) exerts highly potent activity against HIV-1 including multi-drug-resistant variants *in vitro* and *in vivo* [14,16,17]. EFdA has now been forwarded to clinical development in the US.

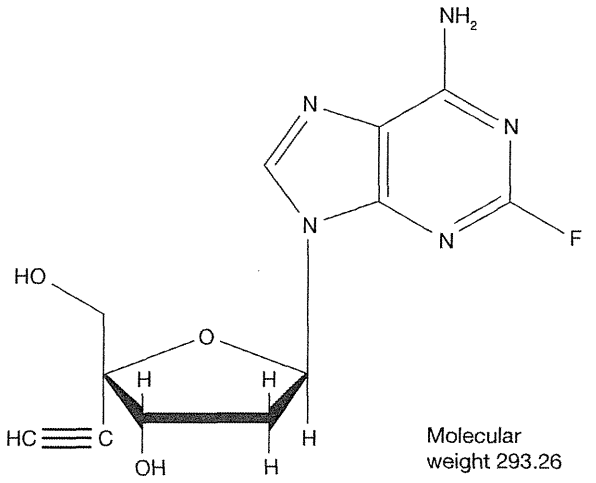
In the present study, we attempted to generate EFdA-resistant HIV-1 variants, employing as a starting HIV-1 population a mixture of 11 highly multiple NRTI-resistant clinical HIV-1 strains (HIV_{11MIX}), isolated from patients with AIDS who had failed multiple antiviral regimens containing various NRTIs. In particular, in the present work, we conducted comparative sequential passages using EFdA, lamivudine (3TC), TDF, FTC, and a recently reported novel RT inhibitor, 2′,3′-didehydro-3′-deoxy-4′-ethynylthymidine (Ed4T; BMS-986001), employing the HIV_{11MIX}.

Methods

Cells, viruses and antiviral agents

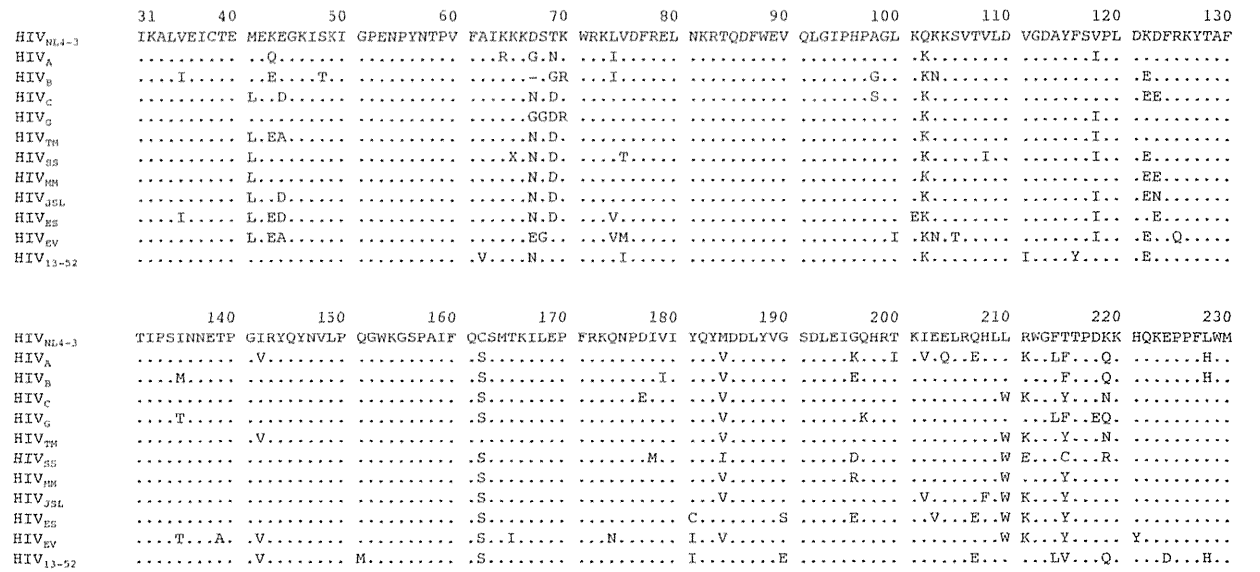
MT-4 cells were grown in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (FCS; Gemini Bio-Products, West Sacramento, CA, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from

Figure 1. Structure of 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA)



HIV-1-seronegative individuals with Ficoll-Hypaque density gradient centrifugation and cultured at a concentration of 10^6 cells/ml in RPMI 1640-based culture medium supplemented with 10% FCS with 10 μg/ml phytohemagglutinin for 3 days prior to use. A total of 11 multi-drug-resistant clinical HIV-1 isolates (HIV_A, HIV_B, HIV_C, HIV_G, HIV_{TM}, HIV_{SS}, HIV_{MM}, HIV_{JSL}, HIV_{ES}, HIV_{EV} and HIV_{13-52}; Figure 2) were isolated from patients who had received antiretroviral therapy containing multiple NRTIs for long periods of time and whose virus strains had acquired a number of substitutions in the RT- and protease-encoding HIV-1 genes [18,19]. HIV_{13-52} was obtained from the HIV and AIDS Reagent Program, NIH (Bethesda, MD, USA). Two recombinant HIV-1 clones ($_{C7}HIV^{EFdA-P10}$ and $_{C9}HIV^{EFdA-P10}$) were propagated using recombinant HIV_{NL4-3}-based infectious molecular clones. Each clone had been introduced with a DNA fragment including 312 nucleic acids in the Gag-coding gene's 3′-end, the entire protease-coding gene, and 951 nucleic acids in the RT-encoding gene's 5′-end) amplified from a drug-selected virus population and digested with *Apa*-I (the Gag-coding region) and *Age*-I (the RT-encoding region). Thus, infectious clones included the entire protease plus the first 312 amino acids in the RT derived from a selected virus population. EFdA was synthesized as previously described [20]. TDF and FTC were obtained from the HIV and AIDS Reagent Program. Ed4T [21] was kindly provided from M Baba (Kagoshima University, Kagoshima, Japan). 3′-Azido-3′-deoxythymidine (AZT) was purchased from Sigma (St Louis, MO, USA).

Figure 2. Amino acid sequences of 11 multi-drug-resistant clinical HIV-1 isolates used for selection



A total of 11 multi-drug-resistant clinical HIV type-1 (HIV-1) isolates (HIV$_{A}$, HIV$_{B}$, HIV$_{C}$, HIV$_{G}$, HIV$_{TH}$, HIV$_{SS}$, HIV$_{MM}$, HIV$_{JSL}$, HIV$_{ES}$, HIV$_{EV}$ and HIV$_{13-52}$) were isolated from patients who had received antiretroviral therapy containing multiple nucleoside reverse transcriptase inhibitors for long periods of time and whose virus strains had acquired a number of substitutions in the reverse transcriptase- and protease-encoding HIV-1 genes [18,19]. HIV$_{13-52}$ was obtained from the HIV and AIDS Reagent Program (NIH, Bethesda, MD, USA).

Antiviral assays

Antiviral assays (MTT assay and p24 assay) using the wild-type HIV-1 (HIV$_{NL4-3}$), a mixture of HIV-1 clinical isolates, and HIV-1 infectious clones that carry amino acid substitution(s) in RT were conducted as previously described [15,19,22,23]. In brief, MT-4 cells were exposed to HIV$_{NL4-3}$ or other HIV-1 strains at 100 50% tissue culture infectious doses (TCID$_{50}$s). After viral exposure, the cell suspension (5×10^3 cells in 100 µl) was plated into each well of a 96-well flat microtitre culture plate containing various concentrations of drugs. After incubation for 5 days, the number of viable cells was determined by the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) and the magnitude of HIV-1 inhibition by anti-HIV agents was determined based on their inhibitory effects of virally induced cytopathicity in MT-4 cells. For p24 assay, MT-4 cells (10^4, 100 µl) or PBMCs (10^5, 100 µl) were exposed to 50 TCID$_{50}$ of each HIV-1 strain and cultured in the presence or absence of various concentrations of drugs in 10-fold serial dilutions in 96-well microculture plates. The amounts of p24 antigen in supernatants were determined on day 5 and the drug concentrations that suppressed the production of p24 Gag protein by 50% (50% inhibitory concentration [IC$_{50}$]) were determined by comparison with the p24 production level in drug-free control cell cultures. All assays were performed in duplicate.

Preparation of a mixture population of highly multi-RT-inhibitor-resistant HIV-1 strains

A total of 11 strains, which contained 9 to 14 amino acid substitutions corresponding to the RT-encoding region, and which have reportedly been associated with HIV-1 resistance to various RT inhibitors, were mixed, propagated in MT-4 cells and the mixed viruses obtained on day 7 of culture was further propagated in fresh MT-4 cells. The culture supernatant containing the mixed viral population (designated HIV$_{11MIX}$) was harvested and used for antiviral assays and drug resistance selection experiments.

In vitro selection of HIV-1 variants resistant to EFdA and other RT inhibitors

The *in vitro* selection of EFdA and other NRTIs was conducted by propagating HIV$_{NL4-3}$ and HIV$_{11MIX}$ in MT-4 cells as previously described [22,24]. In brief, each virus population was propagated in MT-4 cells in the presence of increasing concentrations of each drug. The initial drug concentrations (5 nM to approximately 200 nM) were chosen based on the IC$_{50}$ value of each drug. After 7 days' culture with each drug, the cell-free supernatant containing viruses were harvested, and the viruses were further propagated in fresh MT-4 cells with the same or 2- or 3-fold greater concentration of drug. At the end of each passage, the p24 antigen level in the culture supernatant was measured. When the value was >100 ng/ml, the drug concentration was

increased by 1.5- to 3-fold in the following passage. If the p24 value was 20–99 ng/ml, passage was continued with the same drug concentration and if the value was <20 ng/ml, drug concentration of the next passage was decreased by a factor of approximately 0.7. All selection experiments were concluded when the drug concentration reached 10 μM.

Replication kinetics of HIV_{11MIX} populations

MT-4 cells (10^4, in 96 well) were exposed to 200 $TCID_{50}$ of each HIV_{11MIX} population or recombinant HIV-1 clones ($_{C7}HIV^{EFdA\text{-}P10}$ and $_{C9}HIV^{EFdA\text{-}P10}$) and cultured without antiretroviral agents for 8 days. No fresh MT-4 cells were added for the culture period. The p24 values in supernatants were determined on days 0, 4, 6 and 8. The determination was conducted in duplicate.

DNA sequencing

The cells newly exposed to the cell-free virions during the selection were collected at the end of the indicated passage and lysed for DNA extraction. PCR was performed using primers synthesized according to the selected stretch of the RT-encoding gene. The sequences of the primers were as follows: 5′-GAA CCA AAG AAA GAC TGT TAA GTG TTT CAA-3′ and 5′-ACT CCA TGT ACC GGT TCT TTT AGA-3′. PCR products were cloned into pCR®2.1-TOPO® Vector (Invitrogen, Carlsbad, CA, USA) and transformed in One Shot® MAX Efficiency® DH5α™ T1 Chemically Competent Cells (Invitrogen), followed by plating on lysogeny broth (LB) agar plus ampicillin 100 μg/ml plus X-Gal 40 μg/ml plus IPTG 0.5 mM plates (KD Medical, Columbia, MD, USA) for blue-white colony selection and incubated at 37°C for 16 h. Colonies were picked up, incubated in LB medium, then plasmids were isolated using QIAprep® Spin Miniprep Kit (Qiagen, Germantown, MD, USA). Following plasmid isolation, the sequence analysis in the RT region was conducted using BigDye® Terminator v1.1 (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The following primers were used for the sequencing: 5′-GAC ATA GTC ATC TAT CAA TAC GTG GAT GAT TTG TAT GTA GGA T-3′ and 5′-CAG GAA ACA GCT ATG ACC GCT AGC CCA ATT CAA TTT TCC CAC TAA-3′ and the data were analysed using Sequencher™ 4.9 (Gene Codes, Ann Arbor, MI, USA).

Results

EFdA is highly active against the multi-drug-resistant HIV-1 variants before selection

In the present study, we first determined the activity of EFdA (Figure 1) against the multi-drug-resistant HIV-1 variants before selection. EFdA exerted potent activity, with an IC_{50} of 1 nM in the p24 assay and 0.5 nM in the MTT assay (Table 1), and was 25- to approximately 70-fold more potent than TDF, and 375- to 846-fold more potent than 3TC (Table 1). Subsequently, in order to evaluate the activity of EFdA against highly multi-NRTI-resistant primary HIV-1 strains, HIV_A, HIV_B, HIV_C, HIV_G, HIV_{TM}, HIV_{SS}, HIV_{MM}, HIV_{JSL}, HIV_{ES}, HIV_{EV} and $HIV_{13\text{-}52}$ were employed for assays. Their *pol*-encoding base sequences are illustrated in Figure 2. These HIV-1 isolates were highly resistant to a number of antiviral agents as tested *in vitro* [18,19]. As shown in Table 2, AZT was highly active against the wild type $HIV\text{-}1_{NL4\text{-}3}$ and its IC_{50} value was as low as 0.031 μM; however, IC_{50} values of AZT against six multi-drug-resistant HIV-1 variants (HIV_B, HIV_C, HIV_{TM}, HIV_{EV}, HIV_{JSL} and $HIV_{13\text{-}52}$) ranged from 0.068 to 0.51 μM (Table 2). Ed4T had activity against $HIV_{NL4\text{-}3}$ with an IC_{50} value of 0.43 μM, and its IC_{50} values against these variants ranged from 0.082 to 5.84 μM. By contrast, EFdA inhibited the replication of such variants with IC_{50} values ranging between 0.0002 and 0.15 μM (Table 2). When we determined the activity of each antiretroviral agent against HIV_{11MIX}, 3TC and FTC were virtually inactive with IC_{50} values of >10 μM (Table 1). AZT was active only

Table 1. Anti-HIV type-1 activity of reverse transcriptase inhibitors against wild-type HIV and HIV_{11MIX}^{P0}

Drug	IC_{50} ±SD, μM		
	$HIV_{NL4\text{-}3}$ (MTT assay)	$HIV_{NL4\text{-}3}$ (p24 assay)	HIV_{11MIX}^{P0} (p24 assay; fold difference)[a]
EFdA	0.0005 ±0.0003	0.001 ±0.0009	0.032 ±0.016 (×32)
TDF	0.035 ±0.009	0.025 ±0.003	0.57 ±0.17 (×23)
FTC	0.335 ±0.274	0.360 ±0.252	>10 (>×27)
Ed4T	0.26 ±0.158	0.157 ±0.137	2.6 ±0.94 (×17)
AZT	0.016 ±0.017	0.010 ±0.012	5.1 ±2.6 (×510)
3TC	0.423 ±0.239	0.375 ±0.134	>10 (>×26)

The MT-4 cells were infected with each virus in the presence or absence of drugs and cultured for 7 days. Data was obtained using MTT assay or p24 assay, and 50% inhibitory concentrations (IC_{50}s) were determined. Assays were conducted in triplicate. [a]Values in parentheses refer to the fold change of IC_{50} values compared to wild type ($HIV_{NL4\text{-}3}$, p24 assay). AZT, zidovudine; FTC, emtricitabline; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine.

Table 2. Antiviral activity of EFdA against multi-drug-resistant clinical HIV type-1 isolates

Drug	IC_{50} ±SD, μM (p24 assay; fold difference)[a]						
	HIV_{NL4-3}	HIV_B	HIV_C	HIV_{IM}	HIV_{EV}	HIV_{JSL}	HIV_{13-52}
EFdA	0.0018 ±0.0017	0.15 ±0.07 (×83)	0.023 ±0.009 (×13)	0.0042 ±0.0008 (×2.3)	0.0011 ±0.0002 (×0.6)	0.0015 ±0.0008 (×0.8)	0.0002 ±0.0001 (×0.1)
Ed4T	0.43 ±0.05	4.16 ±2.02 (×9.6)	3.34 ±2.0 (×7.8)	5.02 ±0.92 (×12)	0.74 ±0.27 (×1.7)	5.84 ±1.12 (×14)	0.082 ±0.005 (×0.2)
AZT	0.031 ±0.009	0.43 ±0.08 (×14)	0.35 ±0.06 (×11)	0.33 ±0.11 (×11)	0.51 ±0.13 (×16)	0.65 ±0.23 (×21)	0.068 ±0.023 (×2.2)

Peripheral blood mononuclear cells were infected with each virus with/without different concentrations of drugs in 96 wells, cultured for 7 days. The p24 values in the supernatants were determined and 50% inhibitory concentration (IC_{50}) values were calculated. [a]Values in parentheses refer to the fold changes of IC_{50} values compared to HIV_{NL4-3}. AZT, zidovudine.

at a high concentration with IC_{50} values of 5.1 μM. By contrast, EFdA potently blocked the replication of HIV_{11MIX} with an IC_{50} value of 32 nM (Table 1). Ed4T and TDF were moderately active against HIV_{11MIX} with IC_{50} values of 2.6 and 0.57 μM, respectively, although their IC_{50} values were 17- to approximately 23-fold greater compared to those against the wild-type HIV-1 (HIV_{NL4-3}; Table 1). The IC_{50} value of AZT against HIV_{11MIX}^{P0} (5.1 μM as tested in MT-4 cells) was much greater than those against six multi-drug-resistant clinical isolates (Table 2; 0.068 to 0.65 μM in PBMC).

In vitro selection of HIV-1 variants resistant to EFdA using wild-type $HIV\text{-}1_{NL4-3}$

We attempted to select HIV-1 variants with EFdA by propagating a wild-type laboratory HIV-1 strain, HIV_{NL4-3}, in MT-4 cells in the presence of EFdA. HIV_{NL4-3} was initially exposed to 5 nM EFdA and underwent 25 passages in the presence of increasing concentrations of EFdA up to 500 nM. We simultaneously selected HIV-1 variants in the presence of 3TC, TDF, FTC and Ed4T using the same protocol. As shown in Figure 3A, HIV-1 variants that replicated in the presence of >2 μM 3TC and Ed4T emerged by passage 10, whereas HIV-1 exposed to EFdA and TDF continued to replicate poorly and failed to further replicate in the presence of 100 nM and 500 nM, respectively. The sequence analysis for the RT-encoding region using proviral DNA isolated from the infected MT-4 cells showed that the M184V substitution emerged in the presence of 3TC (by passage 15), K70R with Ed4T (by passage 10) and M184I with EFdA (by passage 10; Figure 3A and Table 3), indicating that the emergence of EFdA- and TDF-resistant HIV-1 variants was substantially delayed compared with other NRTIs; even the virus with EFdA treatment acquired M184I substitution at the early passage, probably because the antiviral activity of EFdA is potent enough to maintain the activity against HIV-1 variant with M184I.

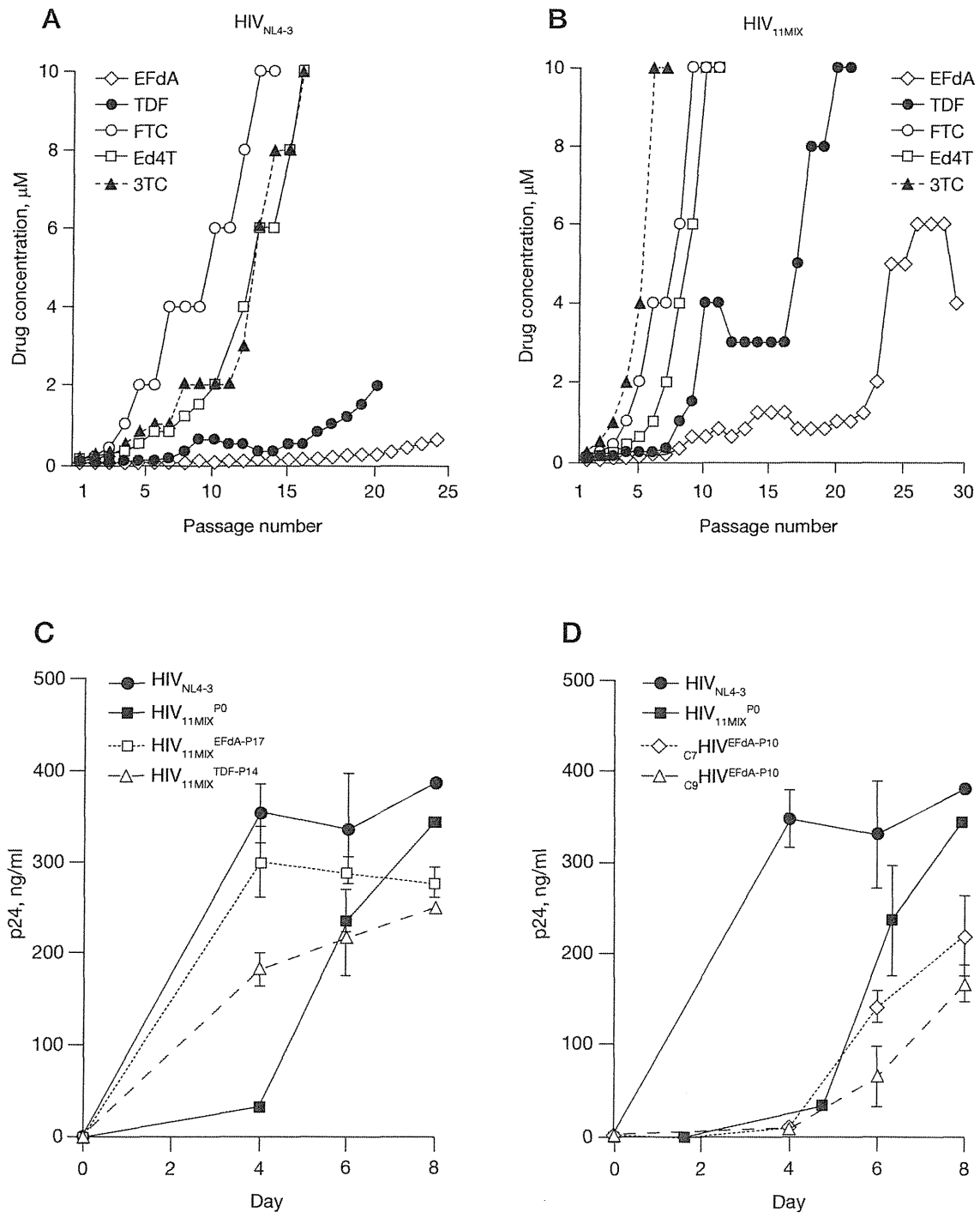
Selection of EFdA-resistant HIV-1 using a mixture of multi-drug-resistant HIV-1 isolates

As described above, when a single wild type HIV-1 strain was used as a starting virus for the selection against EFdA, only one substitution (M184I) emerged by passage 15 and it caused no drastic changes in the activity of EFdA (Figure 3A). Thus, we employed a mixture of 11 HIV-1 clinical isolates resistant to multiple NRTIs as a starting virus population for the selection assay, expecting that HIV-1 acquires drug resistance more expeditiously through homologous recombination among the mixture of 11 HIV-1 isolates [25,26].

The 11 primary HIV-1 strains were mixed and MT-4 cells were exposed to the mixture, the cell supernatant was harvested on day 7, and the obtained viral population was subjected to selection passage. While the same titre of 11 virus isolates were used for the selection, the virus population at the end of passage 0 (HIV_{11MIX}^{P0}) had become comprised of two major isolates, HIV_C (67%) and HIV_B (28%; Figure 2 and Table 3).

When HIV_{11MIX} was further propagated in a cell-free transmission manner in MT-4 cells in the presence of increasing concentrations of each drug, it took only 10 passages for the concentration of EFdA to reach 500 nM (Figure 3B) although it took 24 passages for the EFdA concentration to reach 500 nM in the selection of HIV_{NL4-3} (Figure 3A), demonstrating that HIV_{11MIX} very rapidly acquired the ability to replicate despite the presence of a higher concentration of EFdA than HIV_{NL4-3}. By passage 14, HIV_{11MIX} had become replicative in the presence of 1.2 μM EFdA; however, the virus population failed to replicate in the presence of any further greater concentrations of EFdA until passage 22. Beyond passage 23, EFdA concentration relatively rapidly went up to 6 μM and the virus remained replication-competent by passage 26. However, no significant additional amino acid substitutions were identified in the polymerase-encoding region between passages 20 and 25 (Table 3). As shown in Figure 3B, HIV_{11MIX} failed to replicate in the presence of >6 μM EFdA.

Figure 3. *In vitro* selection of HIV_{NL4-3} and HIV_{11MIX} with NRTIs and replication kinetics of drug-resistant variants



(A) *In vitro* selection of HIV_{NL4-3} resistant to emtricitabine (FTC), lamivudine (3TC), Ed4T, tenofovir disoproxil fumarate (TDF) and EFdA. The selection was carried out in a cell-free manner for a total of 25 passages, with drug concentrations escalating up to 10 μM. (B) *In vitro* selection of HIV_{11MIX} resistant to nucleoside reverse transcriptase inhibitors (NRTIs). A mixture of 11 HIV type-1 (HIV-1) isolates resistant to multiple NRTIs and protease inhibitors was passaged in the presence of increasing concentrations of an NRTI in MT-4 cells. Replication kinetics of (C) EFdA- and TDF-selected HIV-1 variants and (D) recombinant infectious clones were determined in the absence of antiretroviral agents. Each virus population was propagated in MT-4 cells in a 96-well microtitre culture plate for 8 days. No fresh MT-4 cells were added for the culture period. The p24 values in supernatants were determined on days 0, 4, 6 and 8 in duplicate. The replication kinetics of HIV_{NL4-3} is shown in both panels as a reference.

Table 3. Amino acid substitutions in the reverse transcriptase region of HIV_{NL4-3} and HIV_{11MIX} following exposure to EFdA, TDF and Ed4T

Virus	Passage	Amino acid											Ratio, % (clone number)
		M41	E44	D67	T69	K70	L74	V75	M184	L210	T215	K219	
HIV_{NL4-3}	0	–	–	–	–	–	–	–	–	–	–	–	
HIV_{NL4-3}^{EFdA}	15	–	–	–	–	–	–	–	I	–	–	–	100
HIV_{NL4-3}^{TDF}	15	–	–	–	–	–	–	–	–	–	–	–	100
HIV_{NL4-3}^{Ed4T}	15	–	–	–	–	R	–	–	–	–	–	–	100
HIV_{11MIX}^{P0}	0[a]	L	D	N	D	–	–	–	V	W	Y	N	67 (12/18)
	0[a]	–	–	Δ	G	R	I	–	V	–	F	Q	28 (5/18)
	0[a]	–	–	Δ	–	–	–	–	V	–	F	Q	5 (1/18)
HIV_{11MIX}^{EFdA}	5[b]	L	–	Δ	G	R	I	T	V	–	F	Q	67 (8/12)
	5[b]	L	D	N	D	–	–	–	V	W	Y	N	25 (3/12)
	5[b]	L	–	Δ	G	R	I	T	V	W	F	Q	8 (1/12)
	10	L	–	Δ	G	R	I	T	V	–	F	Q	100 (20/20)
	20	L	–	Δ	G	R	I	T	V	–	F	Q	100 (20/20)
	25	L	–	Δ	G	R	I	T	V	–	F	Q	100 (8/8)
HIV_{11MIX}^{TDF}	5	L	–	Δ	G	R	I	T	V	–	F	Q	100 (10/10)
	10	L	–	Δ	G	R	I	A	V	–	F	Q	100 (13/13)
	18	L	–	Δ	G	R	I	–	V	–	F	Q	100 (20/20)
	22	L	–	Δ	G	R	I	–	V	–	F	Q	100 (8/8)
HIV_{11MIX}^{Ed4T}	5[c]	L	D	N	D	–	–	–	V	W	Y	N	80 (8/10)
	5[c]	L	–	Δ	G	R	I	T	V	–	F	Q	20 (2/10)
	10[d]	L	–	Δ	G	R	I	T	V	–	F	Q	60 (3/5)
	10[d]	L	D	N	D	–	–	–	V	W	Y	N	40 (2/5)

Amino acid sequences of proviral DNA were determined using cell lysates of HIV-1-infected MT-4 cells at the termination of each indicated passage. [a]Three apparently major HIV_{11MIX} populations at passage 0 among 18 clones sequenced are illustrated. The primary clone (67%) represents HIV_C and secondary clone (28%) represents HIV_B. [b]Three apparently major HIV_{11MIX}^{EFdA} populations at passage 5 among 12 clones sequenced are illustrated. [c]Two apparently major HIV_{11MIX}^{Ed4T} populations at passage 5 among 10 clones sequenced are illustrated. [d]Two apparently major HIV_{11MIX}^{Ed4T} populations at passage 10 among 5 clones sequenced are illustrated. TDF, tenofovir disoproxil fumarate.

We concurrently conducted the selection under the same conditions using four NRTIs: 3TC, FTC, TDF and Ed4T. As shown in Figure 3B, the concentrations of 3TC, FTC and Ed4T had reached 10 μM by passages 6, 9 and 10, respectively, and the virus was robustly replicating. TDF concentration relatively rapidly reached 4 μM by passage 10, while as in the case of EFdA, the virus failed to replicate in the presence of 4 μM TDF during passage 11 (4 ng/ml p24 in the presence of TDF versus 65 ng/ml p24 without drug); thus, the TDF concentration was decreased to 3 μM and succeeded in continuing the selection. By passage 15 and beyond, HIV_{11MIX} resumed replication, and HIV_{11MIX} started robust replication even in the presence of 10 μM TDF at passage 20 and beyond (Figure 3B).

HIV_{11MIX} selected with EFdA, TDF and Ed4T appeared to have acquired amino acid substitutions/deletions *de novo* and/or through homologous recombination

As shown in Table 3, HIV_{11MIX} at passage 0 (before the selection, designated as HIV_{11MIX}^{P0}), was found to contain three major strains as 18 clones of HIV_{11MIX}^{P0} were individually sequenced. At passage 5, the HIV_{11MIX} population propagated in the presence of TDF (HIV_{11MIX}^{TDF}) was found to contain only one major strain (10/10) that had acquired V75T in RT, which was absent in HIV_{11MIX}^{P0}, but otherwise had a combination of amino acid substitutions identified in HIV_{11MIX}^{P0}. The pattern of amino acid substitutions in HIV_{11MIX}^{TDF} did not change at further passages 10, 18 and 22, although the amino acid at position 75 reverted to the wild-type valine by passage 18 and beyond (Table 3). HIV_{11MIX} selected with Ed4T (HIV_{11MIX}^{Ed4T}), at passage 5, appeared to have remained the same as the major strain in HIV_{11MIX}^{P0}, while HIV_{11MIX}^{Ed4T} had acquired D67 deletion and V75T substitution in 60% of its clones (3/5) at passage 10 but the rest of the clones had the same set of substitutions seen in the major strain of HIV_{11MIX}. In the HIV_{11MIX} selected with EFdA (HIV_{11MIX}^{EFdA}) at passage 5, the predominant strain in HIV_{11MIX}^{P0} had become the second major strain (25%: 3/12 clones). Instead, 75% of the clones (9/12) had acquired V75T substitution, and these 9 clones contained the combination of the substitutions seen in HIV_{11MIX}^{P0}. At passage 10 and beyond, the major strain in HIV_{11MIX} at passage 5 became predominant and all the clones (100%) examined contained the sequence of the major strain.

These data strongly suggested that HIV_{11MIX} selected with EFdA, TDF and Ed4T acquired amino acid substitutions/deletions *de novo* and through homologous recombination among multiple viral species within HIV_{11MIX}, although it was also possible that the V75T and V75A, both of which were not seen in HIV_{11MIX}^{P0}, arose from minor species within HIV_{11MIX}.

HIV_{11MIX} populations selected with EFdA and TDF remained relatively sensitive to EFdA

As shown in Table 1, HIV_{11MIX}^{P0} was highly resistant to 3TC, AZT and FTC with IC_{50} values of >5 μM. HIV_{11MIX}^{P0} was still susceptible to Ed4T, TDF and EFdA with IC_{50} values of 2.6, 0.57 and 0.032 μM. By contrast, the HIV_{11MIX} populations selected with EFdA and TDF and harvested at passages 17 and 14 (designated $HIV_{11MIX}^{EFdA\text{-}P17}$ and $HIV_{11MIX}^{TDF\text{-}P14}$) were highly resistant to FTC, Ed4T, AZT and 3TC with IC_{50} values of >10 μM. These two variants, $HIV_{11MIX}^{EFdA\text{-}P17}$ and $HIV_{11MIX}^{TDF\text{-}P14}$, were also resistant to TDF with IC_{50} values of 8 and >10 μM, respectively. However, $HIV_{11MIX}^{EFdA\text{-}P17}$ and $HIV_{11MIX}^{TDF\text{-}P14}$ remained susceptible to EFdA with IC_{50} values of 0.15 and 0.1 μM, although their IC_{50} fold-differences relative to the IC_{50} values against $HIV_{NL4\text{-}3}$ were 150 and 100, respectively (Table 4).

The amino acid substitutions in the *pol*-encoding gene are responsible for HIV_{11MIX}'s acquisition of resistance to the NRTIs examined

In an attempt to determine whether the amino acid substitutions identified in the *pol*-encoding gene were responsible for the observed drug resistance, we generated two replication-competent recombinant HIV-1 clones, ${}_{C7}HIV^{EFdA\text{-}P10}$ and ${}_{C9}HIV^{EFdA\text{-}P10}$, by introducing the *pol*-encoding gene excised from an EFdA-selected variant HIV_{11MIX}^{P10} into a plasmid $pHIV_{NL4\text{-}3}$. The only difference between ${}_{C7}HIV^{EFdA\text{-}P10}$ and ${}_{C9}HIV^{EFdA\text{-}P10}$ was an absence of the M41L substitution in the latter. Both recombinant clones proved highly resistant against FTC, Ed4T, AZT and 3TC with IC_{50} values of approximately ≥10 μM (Table 4). They were also substantially resistant to TDF with IC_{50} values of 2.6 to approximately 3.2 μM, while both clones were yet susceptible to EFdA with IC_{50} values of 0.055 to approximately 0.05 μM (Table 4). These results indicated that the amino acid substitutions acquired in the *pol*-encoding gene were associated with the development of resistance to all the NRTIs and that EFdA continued to exert substantial activity to HIV variants harbouring such amino acid substitutions.

A single amino acid substitution in RT reduces the anti-HIV activity of TDF, FTC, AZT and 3TC but not the activity of EFdA or Ed4T

HIV_{11MIX}, following the selection with TDF, Ed4T and EFdA, acquired various amino acid substitutions (Table 3). Among those substitutions, M41L, K70R, T215F and K219Q cause high-level resistance against various currently approved NRTIs and have been termed as thymidine-analogue mutations (TAMs). Thus, we examined the effects of a single or combination of such mutations on the activity of various NRTIs including EFdA using three newly generated recombinant clones, $HIV_{NL4\text{-}3}^{M184V}$, $HIV_{NL4\text{-}3}^{K65R}$ and $HIV_{NL4\text{-}3}^{D67del/T69G/K70R}$.

As shown in Table 5, $HIV_{NL4\text{-}3}^{M184V}$ was resistant to FTC and 3TC, was moderately resistant to TDF, but was susceptible to AZT, Ed4T and EFdA. The K65R substitution, which was not selected in the present study but has been reportedly associated with HIV-1 resistance against TDF, 3TC and FTC, was also examined for its effects on the alterations of the antiretroviral activity of EFdA. The K65R substitution reduced the antiretroviral activity of TDF, FTC and 3TC by 34-, 9- and 12-fold, respectively (Table 5) and also attenuated AZT's antiretroviral activity by 2.3-fold. However,

Table 4. Anti-HIV type-1 activity of various nucleoside reverse transcriptase inhibitors against HIV_{11MIX}, HIV_{11MIX}^{EFdA}, HIV_{11MIX}^{TDF} and recombinant variants

	IC_{50} ±SD, μM (p24 assay; fold difference)[a]			
	Passage virus		Recombinant clone	
Drug	$HIV_{11MIX}^{EFdA\text{-}P17}$	$HIV_{11MIX}^{TDF\text{-}P14}$	${}_{C7}HIV^{EFdA\text{-}P10}$	${}_{C9}HIV^{EFdA\text{-}P10}$
EFdA	0.15 ±0.035 (×150)	0.1 ±0.01 (×100)	0.055 ±0.033 (×55)	0.05 ±0.025 (×50)
TDF	8 ±4.1 (×320)	>10 (>×400)	2.6 ±1.6 (×104)	3.2 ±1.8 (×128)
FTC	>10 (>×27)	>10 (>×27)	>10 (>×27)	>10 (>×27)
Ed4T	>10 (>×63)	>10 (>×63)	9.6 ±3.7 (×61)	>10 (>×63)
AZT	>10 (>×1,000)	>10 (>×1,000)	>10 (>×1,000)	>10 (>×1,000)
3TC	>10 (>26)	>10 (>×26)	>10 (>×26)	>10 (>×26)

MT-4 cells were used for the assay. The $HIV^{EFdA\text{-}P10}$ clones had $HIV_{NL4\text{-}3}$ sequence as a backbone but had the *pol* sequence from DNA isolated from cells infected with selection viruses (see the *Methods* section). Clone number 7 (${}_{C7}HIV^{EFdA\text{-}P10}$) had the same mutations as shown in Table 4 ($HIV_{11MIX}^{EFdA\text{-}P10}$), while ${}_{C9}HIV^{EFdA\text{-}P10}$ had the same mutations except for M41 (M41wt). [a]The numbers in parentheses refer to the fold changes of 50% inhibitory concentration (IC_{50}) values compared to $HIV_{NL4\text{-}3}$ in the p24 assay shown in Table 1. AZT, zidovudine; FTC, emtricitabline; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine.