We suggest that unknown cellular function(s) closely linked to these sequence motifs may be necessary for the co-receptor activity of GPCRs.

Thus, GPCRs harbouring tyrosines in the NTR have the potential of HIV/SIV co-receptors, and their co-receptor activity should be examined. HIV-1 has been expanding its ability to use other GPCRs with tyrosines in the NTR as novel co-receptors, so future studies are likely to identify novel co-receptors. There is no evidence that CCR5 and CXCR4 are the only co-receptors that play important roles in HIV-1 infection *in vivo* and progression of AIDS. The contributions of additional co-receptors such as D6, FPRL1 and GPR1 in HIV-1 infection *in vivo* should be elucidated further.

ACKNOWLEDGEMENTS

This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labour and Welfare of Japan and the 21st Century COE Program, 'Biomedical Research using Accelerator Technology', Gunma University Graduate School of Medicine, Gunma, Japan.

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Characterization of a CD4-independent clinical HIV-1 that can efficiently infect human hepatocytes through chemokine (C-X-C motif) receptor 4

Peng Xiao[a,b], Osamu Usami[a], Yasuhiro Suzuki[a], Hong Ling[b], Nobuaki Shimizu[c], Hiroo Hoshino[c], Min Zhuang[b], Yugo Ashino[a], Hongxi Gu[b] and Toshio Hattori[a]

Objective: HIV-1 isolates are prominently CD4-dependent and, to date, only a few laboratory-adapted CD4-independent strains have been reported. Therefore, whether CD4-independent viruses may exist in HIV-1-infected patients has remained unclear. Here, we report the successful isolation of a CD4-independent clinical HIV-1 strain, designated SDA-1, from the viral quasispecies of a therapy-naive HIV-1 and *Pneumocystis jirovecii* pneumonia patient in the late-stage of AIDS with extremely low CD4 cell count (CD4 = 1/μl). We characterized this virus and further explored whether it could infect or induce pathological effects in human hepatocytes.

Design and methods: To determine coreceptor usage and CD4-independent infection, the HIV-1 envelope (Env)-pseudotypes and Env-chimeric viruses were used.

Results: SDA-1 was able to infect $CD4^-$ cell lines through either chemokine (C-X-C motif) receptor 4 or CCR5. It still maintained the ability to infect $CD4^+$ cells through multiple coreceptors of chemokine (C-X-C motif) receptor 4, chemokine (C-C motif) receptor 5, chemokine (C-C motif) receptor 3 and chemokine (C-C motif) receptor 8. Productive infection by SDA-1 was noted in both CD4-negative hepatoma cells and primary cultured human hepatocytes. Moreover, we demonstrated that SDA-1 could efficiently infect human hepatocytes on both static and mitotic phases through chemokine (C-X-C motif) receptor 4, without inducing apoptotic cell death.

Conclusion: The present study provides evidence that emergence of CD4-independent HIV-1 virus *in vivo* may occur in HIV-1-infected patients. In addition, these results shed light on the mechanisms involved in liver damage in HIV-1-infected individuals, which could have important implications concerning the range of mutability and the pathogenesis of AIDS. © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins

AIDS 2008, **22**:1749–1757

Keywords: CD4-independence, HIV-1, human hepatocytes, human hepatoma cells

Introduction

The entry of HIV-1 into target cells requires interaction of the viral envelope (Env) with CD4 and a chemokine coreceptor [1,2]. Macrophage-tropic HIV-1 viruses primarily use chemokine (C-C motif) receptor 5 (CCR5) (R5) as a coreceptor, whereas T-cell-tropic viruses use chemokine (C-X-C motif) receptor 4

[a]Department of Emerging Infectious Diseases, Division of Internal Medicine, Graduate School of Medicine, Tohoku University, Sendai, Japan, [b]Department of Microbiology, Harbin Medical University, Harbin, China, and [c]Department of Virology and Preventive Medicine, Gunma University School of Medicine, Gunma, Japan.

Correspondence to Toshio Hattori, MD, PhD, Department of Emerging Infectious Diseases, Division of Internal Medicine, Graduate School of Medicine, Tohoku University, Sendai 980-8574, Japan.

Tel: +81 22 717 8220; fax: +81 22 717 8221; e-mail: hattori.t@rid.med.tohoku.ac.jp
Received: 17 January 2008; revised: 21 April 2008; accepted: 9 May 2008.

DOI:10.1097/QAD.0b013e328308937c

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(CXCR4) (X4). Dual-tropic viruses (R5X4) use both coreceptors [3]. A few rare viruses can also use alternative coreceptors such as chemokine (C-C motif) receptor 1 (CCR1), chemokine (C-C motif) receptor 2b (CCR2b), chemokine (C-C motif) receptor 8 (CCR8), chemokine (C-X-C motif) receptor 6 (CXCR6), G protein-coupled receptor 1 (GPR1) or GPR15/Bob for entry into coreceptor-transfected $CD4^+$ cell lines [4]. Whatever the coreceptor specificity of an HIV-1 isolate, an interaction with CD4 is always the first step in a chain of events leading to fusion of the viral envelope with the cellular membrane. However, previous studies have shown that SIV [5] and HIV-2 [6] can also infect cells independently of CD4.

In contrast to SIV and HIV-2, HIV-1 CD4-independent viruses are rarely isolated. To date, only a few laboratory CD4-independent HIV-1 variants [7–10] have been reported. Therefore, whether such viruses may exist in HIV-1-infected patients has remained unclear. However, several studies [11–14] have shown that HIV-1-DNA and p24, a core HIV-1 antigen, were detected in CD4-negative cells or tissues such as brain, kidney and liver in HIV-infected individuals, suggesting the possibility that low levels of CD4-independent variants exist *in vivo*. Among such $CD4^-$ cells or tissues, liver is an important organ in determining the prognosis of HIV-1-infected patients. End-stage liver disease is becoming a frequent cause of death in HIV-1-infected hospitalized patients [15–17]. Although the cause of liver injury in HIV-1 patients might be multifactorial, such as hepatitis B virus (HCV) and hepatitis B virus (HBV) coinfection and the side effects of antiretroviral therapy, a number of reports have documented that histological liver abnormalities occurred solely as a result of HIV-1 infection [13,18,19]. Nonetheless, few attempts have been made to elucidate the mechanisms of the liver damage in HIV-1-infected individuals.

In this study, we successfully isolated a CD4-independent clinical HIV-1 strain, designated SDA-1, from the viral quasispecies of a therapy-naive HIV-1 and *Pneumocystis jirovecii* pneumonia (PJP) patient in the late stage of AIDS with extremely low CD4 cell numbers. We characterized the phenotype of this virus and further explored whether it could infect or induce pathological effects in human hepatocytes.

Materials and methods

Patient's information

A 53-year-old Japanese man infected with HIV-1 was admitted to Tohoku University Hospital owing to prolonged fever and severe dyspnea in 2000. His plasma viral load and CD4 cell count at the time of admission was 220 000 copies/ml and 1 cell/μl, respectively. He was diagnosed with PJP, and his clinical stage was classified as IV-C3 [20]. The onset and route of HIV-1 infection were unknown. No evidence of coinfection with HBV or HCV in this patient was found. The patient was treated with trimethoprim and sulfamethoxazole (TMP–SMX) and highly active antiretroviral therapy (HAART). His condition deteriorated rapidly and he died 33 days after admission. Consent for autopsy was denied by the patient's family.

Before HAART, plasma samples and peripheral blood mononuclear cells (PBMC) were collected from this patient and cryopreserved in liquid nitrogen until use. The institutional Ethics Committee approved this study and written informed consent was obtained from the patient.

Virus isolation

HIV-1 isolation was achieved by using an in-vitro short-term phytohemagglutinin (PHA)–PBMC coculture method. Briefly, cryopreserved PBMC (2×10^6) from the patient were cocultivated with PHA-stimulated PBMC (5×10^6) from an HIV-1 seronegative healthy donor. The culture was maintained in RPMI-1640 (Invitrogen, California, USA) containing 10% fetal calf serum and 5 U/ml of recombinant interleukin-2 (IL-2) (Sigma, St. Louis, Missouri, USA). Proliferation of HIV-1 was examined by measuring p24 antigen in the cell culture supernatant using a p24 ELISA kit (RETRO-TEK, ZeptoMetrix Corp., New York, USA). The virus stocks were kept at −80 °C until use.

Amplification of *env* and sequence analysis

The full-length HIV-1 *env* genes were amplified by limiting dilution nested PCR from proviral PBMC DNA or plasma RNA as previously described [21,22]. To avoid artificial recombination and resampling of the viral genomes, independent nested PCR reactions were carried out per specimen [23,24].

The first round PCR was conducted with a F5852–R8935 primer pair (F5852, 5′-TAGAGCCCTGGAAG CATCCAGGAAG, HIV-1 HXB2 nucleotide position 5852–5876; R8935, 5′-TTGCTACTTGTGATTGCT CCATGT, HXB2 nucleotide position 8912–8935). The second round PCR was performed with a F5957–R8903 primer pair (F5957, 5′-GATCGAATTCTAGGCATC TCCTATGGCAGGAAGAAG, HXB2 nucleotide position 5957–5982, containing an additional *Eco*RI site (underlined) to facilitate cloning; R8903, 5′-AGCT CTC GAGGTCTCGAGATACTGCTCCCACCC, HXB2 nucleotide position 8881–8903, containing an additional *Xho*I site (underlined)). The purified PCR products were subcloned into the *Eco*RI and *Xho*I sites of the pSM-HXB2 plasmid. All correctly oriented *env* clones were then screened for biological function [22] followed by sequencing and phylogenetic analysis as previously described [25,26].

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Cell lines and cell culture

All the cell lines, unless otherwise specifically mentioned, were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal calf serum. Human glioma NP-2-CD4$^+$ cells transfected with a variety of chemokine receptors as indicated [27] were maintained in medium containing 500 μg/ml of G-418 (Promega, Wisconsin, USA) and 1 μg/ml of puromycin (Sigma). Human CD4-negative osteosarcoma (HOS) cells expressing either CXCR4 or CCR5 [28] were cultured in medium containing 1 μg/ml of puromycin. Human hepatoma cells Huh-7 and Hep-G2 [29] were obtained through the Cell Resource Center for Biomedical Research, Tohoku University, Japan. Human primary cultured hepatocytes (p-hepatocytes, BD Bioscience, California, USA) were maintained on BD Matrigel with Hepato-STIM hepatocyte culture medium (BD Bioscience).

Reagents and antibodies

The CXCR4 antagonist AMD3100 [30], and the CCR5 antagonist TAK-779 [31] were provided by the NIH AIDS Research and Reference Reagent Programme and Takeda Chemical Industries, Ltd., Osaka, Japan, respectively. Recombinant human soluble CD4 (sCD4) was from ImmunoDiagnostics, Inc. (Woburn, Massachusetts, USA). Antialbumin-fluorescein isothiocyanate (FITC) antibody was from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Anticytokeratin-18–phycoerythrin and antialpha fetoprotein (AFP)–FITC antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). Anti-HIV-1-p24 (clone KC57)-FITC antibody was from Beckman Coulter. All other antibodies were from BD Pharmingen (San Diego, California, USA).

Pseudotyped virus infection assay

The HIV-1 Env-pseudotypes were generated as previously described [32]. Briefly, 293T cells (5×10^6 cells/10 cm-dish) were transfected with 5 μg of luciferase-expressing pNL4-3-Luc-R$^-$E$^-$ [33] or green fluorescent protein (GFP)-expressing pNL4-3-GFP [34] plasmid in combination with 10 μg of one of the *env*-expressing plasmids, pSM-SDA-1, pSM-HXB2 (X4), pSM-ADA (R5), or pSM-89.6 (R5X4). The vesicular stomatitis virus-G pseudotypes were also prepared [35].

For infection assays of luciferase-pseudotypes (luc-p), 10 ng p24 of luc-p were added into each well of 24-well plates (5×10^4 cells/well). After 12 h infection, the cells were washed and incubated for an additional 36 h at 37 °C. The cells were then lysed using a Luciferase Assay kit (Promega) and the luciferase activity was examined by a luminometer (Lumat 9507, Germany). To determine the effects of various reagents related to the viral receptors, target cells were preexposed for 1 h with the indicated concentration of the antagonists, or the antibodies. For GFP-pseudotypes (GFP-p) infection, target cells were infected with 10 ng p24 of GFP-p virus for 48 h and fixed by 5% paraformaldehyde. Infectivities were visualized under a Zeiss LSM510 confocal microscopy and DIC images with a 512 × 512 resolution were acquired.

Chimeric viruses

All *env* recombinant chimeric viruses in this study were generated in the background of pNL43, an X4-tropic HIV-1 infectious clone [36]. Briefly, the fragment of pNL43 containing *Eco*RI (nt 5743–5748) and *Kpn*I (nt 6343–6348) was amplified by PCR with a F5671–R6472 primer pair (F5671, 5′-GGCTCCATAACTTAGGA CAAC, pNL43 nucleotide position 5671–5691; R6472, 5′-TACTTCTTGTGGGTTGGGGTC, pNL43 position 6452–6472), followed by insertion into the pSM–SDA-1 using *Eco*RI and *Kpn*I. The new *Eco*RI–*Xho*I fragment (3155 bp) covering the entire SDA-1 *env* gene was then replaced with the equivalent region of pNL43 to construct the Env-chimeric virus NL43_SDA-1. Similarly, Env-chimeras of ADA (NL43_ADA), 89.6 (NL43_89.6) or truncated *env* (NL43_env (-)) were created, respectively. All Env-chimeric viruses were prepared by transfecting 293T cells as described above. For infection assays, 100 ng p24 of the chimeric viruses or virus stock supernatants were added in each well of 24-well plates (5×10^4 cells/well). After 2 h adsorption, the cells were washed and incubated for 48 h. Viral replication was monitored by p24 antigen production.

Flow cytometry and apoptosis assay

We performed cell-surface staining for CD4, CXCR4 and CCR5 by flow cytometry. To determine the purification and differentiation of p-hepatocytes, we tested the specific markers using antialbumin–FITC, anti-AFP–FITC and anticytokeratin-18–phycoerythrin antibodies. Appropriate class matched antibodies were used in each experiment. To detect the proliferation and intracellular p24, p-hepatocytes were fixed and permeabilized using a Cytofix-Cytoperm kit (BD Bioscience). Subsequently, the cells were stained with anti-Ki-67–phycoerythrin and antip24–FITC antibodies. Apoptosis of the p-hepatocytes was determined using the Apoptosis Detection kit I (BD Pharmingen). Flow cytometry analysis was performed using FACSCalibur (Becton Dickinson, New Jersey, USA). All Data were acquired and analyzed using Cell Quest software (BD Bioscience).

Nucleotide sequence accession number

The GenBank accession number for the sequence determined in this study is AY902478 (SDA-1).

Results

Evaluation of SDA-1 viral quasispecies

In an attempt to isolate CD4-independent clinical HIV-1 strain(s), we performed virus isolation from a

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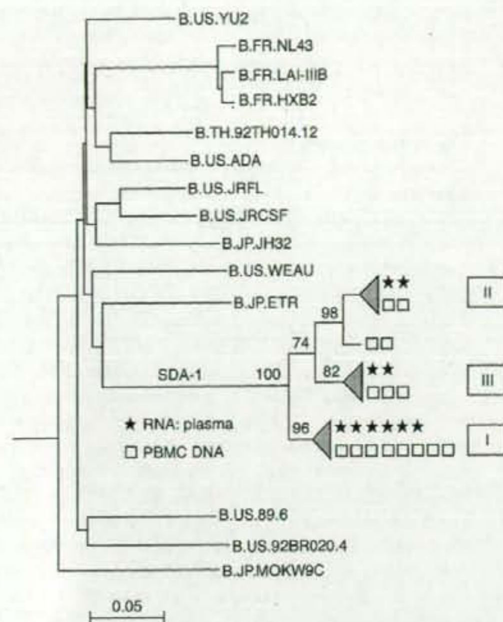

Fig. 1. Evolution of SDA-1 *env* quasispecies in plasma and PBMC. Phylogenetic analysis of newly characterized, SDA-1 gp120 *env* nucleotide sequences obtained from plasma ($n = 10$) and PBMC ($n = 15$) with representative sequences of HIV-1 subtype B. Numbers at branch nodes refer to the percentage of bootstrap values and symbols indicate individual clones.

therapy-naive HIV-1 and PJP patient with extremely low CD4 cell number, and successfully isolated the virus (peak of p24, 500 ng) from this patient and designated it SDA-1. To assess the quasispecies diversity present in *vivo*, we analyzed the SDA-1 *env* clones derived from plasma RNA and PBMC. As shown in Fig. 1, SDA-1 is grouped within the HIV-1 subtype B reference sequences. Within SDA-1's sequence cluster, three phylogenetic forms were identified. Supported by a significant bootstrap value (96%), form I was the predominant quasispecies, representing 70% of all sequences. Two minor quasispecies (forms II and III) had similar structures but differed in the position of the first breakpoint. The mean distances between major and minor quasispecies did not differ significantly from the sequence heterogeneity. Furthermore, the quasispecies diversities between plasma and PBMC were similar within each form, and were all below 5.0%.

Multicoreceptor usage and CD4-independent entry of SDA-1

To determine the receptor usage of SDA-1, we randomly selected five clones from the predominant quasispecies and generated Env-pseudotypes and Env-chimeric viruses as representatives. As a control, the Envs from a variety of HIV-1 subtypes with X4 (HXB2), R5 (ADA), and R5X4 (89.6) tropism were used. Utilizing luciferase-pseudotypes (luc-p), we first examined the coreceptor usage of SDA-1. We found that in the presence of CD4, all representative SDA-1 Env-pseudotypes were able to use efficiently both CXCR4 and CCR5, with additional moderate usage of CCR3 and CCR8 (Fig. 2a).

We next investigated whether SDA-1 Envs are capable of inducing CD4-independent infection. We found that SDA-1 Envs mediated entry into both HOS–CXCR4 and HOS–CCR5. However, the infectivities of SDA-1 for HOS–CXCR4 were approximately 2.5-fold higher than that for HOS–CCR5 (Fig. 2b). In stark contrast, none of the other types of luc-p viruses entered either of those cells. Furthermore, we evaluated the ability of SDA-1 Envs in mediating cell–cell fusion, a dye-transfer cell–cell fusion assay [37] was used with HOS–CXCR4 and HOS–CCR5 cells. Only in the cells expressing SDA-1 Envs (effector cells) did cell–cell fusion with CD4-negative, CXCR4- or CCR5-positive HOS cells (target cells) occur (data not shown).

In addition to the results with HOS–CXCR4 and CCR5, preexposure of HOS cells to Leu-3A, a CD4 monoclonal antibody (mAb) that recognizes the gp120 binding site on CD4 [38], failed to block SDA-1 infection. In contrast, pretreatment with antagonists for CXCR4 or CCR5 effectively inhibited infection (Table 1). Furthermore, the infectivities of SDA-1 on HOS–CXCR4 and HOS–CCR5 were enhanced by preexposure of the virus to sCD4 indicating that the binding of SDA-1 Env to CD4 induces further conformational changes in gp120 to fully expose the chemokine receptor binding domain. Collectively, SDA-1 Envs mediated the CD4-independent infection via both CXCR4 and CCR5.

Having clarified that SDA-1 is a CD4-independent isolate, we next investigated what types of $CD4^-$ cells are able to support SDA-1's entry. We focused first on human liver-derived cell lines, as the mechanisms of the liver damage in HIV-1-infected individuals are still unclear.

Two hepatoma cell lines, Huh-7 and Hep-G2, were used as targets. We first examined the expression of the receptors on the cell surface by flow cytometry and found that both CXCR4 and CCR5 were expressed on Huh-7 and Hep-G2 cells. In contrast, CD4 was not detected on either, which was confirmed by RT–PCR (data not shown). We then evaluated whether SDA-1 can enter into hepatoma cells with luc-p viruses. We found that only SDA-1 luc-p viruses efficiently infected Huh-7; however, its infectivity was marginal in Hep-G2 (Fig. 2c). Previous studies have shown that few HIV-1 variants can infect $CD8^+$ cells using CD8 as receptor [10,39]. Therefore, we further explored receptors used by

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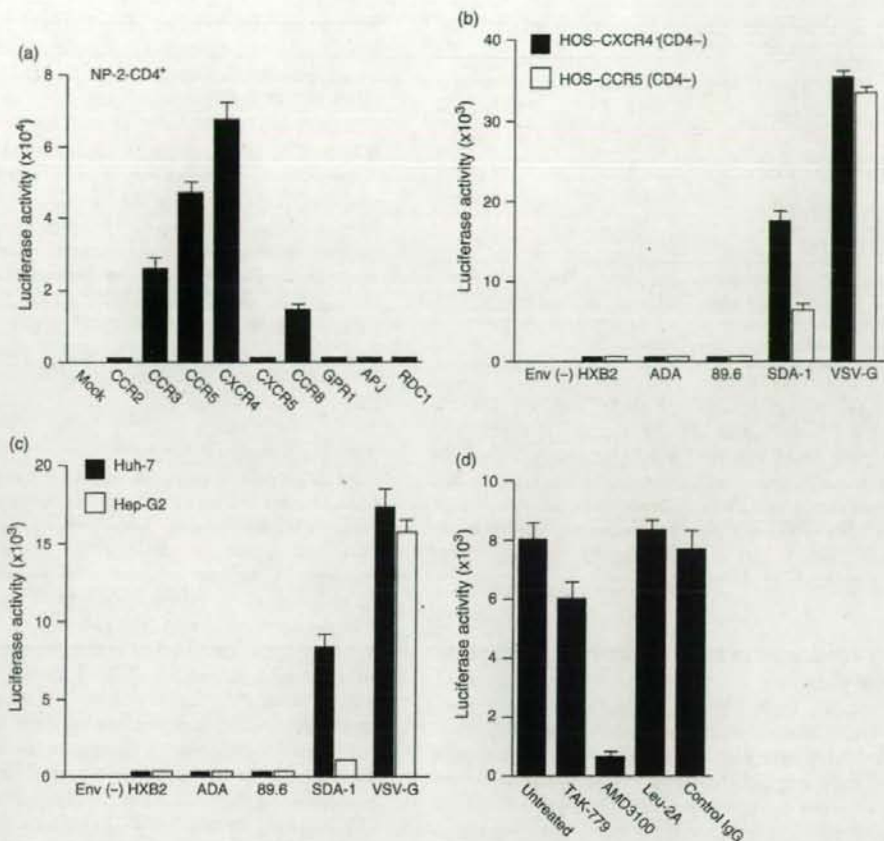

Fig. 2. Multicoreceptor usage and CD4-independent entry of SDA-1. (a) SDA-1 Envs mediate entry of $CD4^+$ cells using multiple coreceptors. NP-2-$CD4^+$ cells coexpressing one of the indicated chemokine receptors were exposed to SDA-1 luc-p viruses for 48 h and the luciferase activities were measured. (b) SDA-1 Envs mediate entry of $CD4^-$ cell lines through either CXCR4 or CCR5. The HOS cells ($CD4^-$) expressing either CXCR4 or CCR5 were exposed to the indicated HIV-1 luc-p viruses or VSV-G for 48 h, after which the infectivities were determined. (c) Entry of SDA-1 into $CD4^-$ human hepatoma cells. Huh-7 and Hep-G2 were exposed to the indicated HIV-1 luc-p viruses or VSV-G. Infectivities were determined at 48 h. (d) Effects of receptor-related antagonists or antibodies on the entry of SDA-1 entry into Huh-7 cells. Interaction of SDA-1 luc-p viruses with Huh-7 cells was tested in the absence or presence of AMD3100 (1.0 μM), TAK-779 (100 nM), anti-CD8 Leu-2A antibody (30 μg/ml) or class-matched control antibody (30 μg/ml). Results shown (a–d) are means of triplicate experiments. Bars, standard deviation. IgG, immunoglobulin G; VSV, vesicular stomatitis virus.

SDA-1 for entry into hepatoma cells. As shown in Fig. 2d, preexposure of Huh-7 to anti-CD8 Leu-2A mAb, as well as the CCR5 antagonist, TAK-779, failed to block SDA-1 infection of Huh-7, whereas anti-CXCR4 with AMD3100 effectively suppressed the infectivity. These results suggested that SDA-1 enters Huh-7 cells principally via CXCR4.

Replication of SDA-1 in human hepatoma cells

Although SDA-1 luc-p viruses infected some cells independently of CD4 cells, it was necessary to determine whether SDA-1 can replicate in those $CD4^-$ cells, particularly in hepatoma cells. For this purpose, we constructed NL43-based Env-chimeric viruses described above. We then examined whether the chimeric viruses were able to replicate in $CD4^-$ cells. As shown in Fig. 3a, the SDA-1 Env-chimeric viruses replicated efficiently in HOS–CXCR4 and HOS–CCR5 cells to similar levels. In contrast, none of the other Env-chimeric viruses infected either of those cells lines. Furthermore, we examined whether SDA-1 Env-chimeric viruses could replicate in hepatoma cells. As shown in Fig. 3b, high levels of NL43-SDA-1 replication were observed in Huh-7 cells. However, marginal replication was detected

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Table 1. Inhibition of SDA-1 by blocking reagents in CD4⁻ cells.

Reagent	% Inhibition	
	HOS–CXCR4	HOS–CCR5
Medium	0	0
Control mAb (30 μg/ml)	0	0
Leu-3A (30 μg/ml)	10	12
Soluble CD4 (10 μg/ml)	225[a]	120[a]
AMD3100 (1.0 μM)	99	0
TAK-779 (100 nM)	0	97

CCR5, chemokine (C-C motif) receptor 5; CXCR4, chemokine (C-X-C motif) receptor 4; HOS, Human CD4-negative osteosarcoma; mAb, monoclonal antibody.
[a]Enhancement of entry.

in Hep-G2 cells. Although both Huh-7 and Hep-G2 cells are derived from human hepatoma, many potential host factors [40] could influence HIV replication, which for the most part remain unknown. Similarly, only Huh-7 cells, but not Hep-G2 cells, were susceptible to HCV [41,42]. These reasons may be related to the difference between Huh-7 and Hep-G2 regarding the level of replication by SDA-1.

SDA-1 replicates in both proliferating and static hepatocytes

To investigate further whether normal human hepatocytes could sustain entry and replication of SDA-1, p-hepatocytes were used for the following experiments. Among the three specific markers of human hepatocytes, both albumin and cytokeratin-18, but not alpha-fetoprotein were detected in the p-hepatocytes suggesting that the hepatocytes we used were well differentiated (data not shown). We also found that CXCR4 was expressed on the surface of p-hepatocytes. In contrast, neither CD4 nor CCR5 was detected on the p-hepatocyte surface or by real-time PCR (RT–PCR) (data not shown).

We next explored whether SDA-1 can enter p-hepatocytes by using GFP-p. As shown in Fig. 4a, only SDA-1 GFP-p viruses gave GFP-positive cells in p-hepatocytes, whereas other HIV-1 GFP-p viruses did not. The GFP-positive cells showed spindle-like shapes suggesting that the infection occurred in the p-hepatocytes but not in the contaminating lymphocytes. Furthermore, we studied whether SDA-1 can replicate in the p-hepatocytes. As shown in Fig. 4b, the p-hepatocytes were productively infected by the SDA-1 Env-chimeric viruses and SDA-1 virus stock itself but not by the other HIV-1 Env-chimeric viruses. Moreover, we found that AMD3100 inhibited the replication of SDA-1 in p-hepatocytes in a dose-dependent manner (Fig. 4c) indicating that the infection of p-hepatocytes by SDA-1 was mediated through CXCR4.

A previous study [19] reported that the HIV-1 gp120 *env* directly caused hepatocyte death by signaling through CXCR4 *in vitro*; however, most studies were performed using the hepatoma Huh-7 cells not hepatocytes, therefore, it may not really reflect the nature of liver damage. To explore the pathological effects of HIV-1 CD4-independent infection on hepatocytes, we exposed p-hepatocytes to the SDA-1 and analyzed cell viability. We found that the viability of the p-hepatocytes in cells cultured with or without SDA-1 Env-chimeric viruses was comparable (96%, P was not significant) indicating that HIV-1 CD4-independent infection rarely induces hepatocyte death via an apoptotic process (data not shown). To further examine whether the infection or replication of SDA-1 is limited only to a certain number of p-hepatocytes or whether the infectivity or replication is influenced by the cell cycle, we studied the intracellular expression by flow cytometry of p24 and Ki-67 [43], a marker strictly associated with cell proliferation, in the HIV-1-infected p-hepatocytes. As shown in Fig. 4d, we found that 32.49% of p-hepatocytes were infected by SDA-1. However, there was no significant difference in

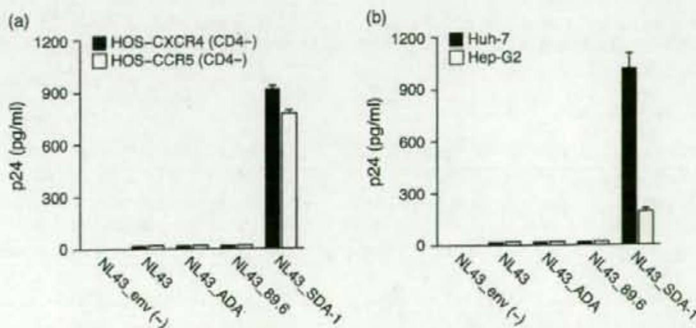

Fig. 3. CD4-independent infection of SDA-1 Env-chimeric viruses. The HOS cells (CD4⁻) expressing either CXCR4 or CCR5 (a) and two CD4⁻ human hepatoma cells (b) were incubated with the indicated HIV-1 Env-chimeric viruses. Virus replication was then monitored by p24 antigen production on day 3. Results shown (a, b) are means of triplicate experiments. Bars, standard deviation.

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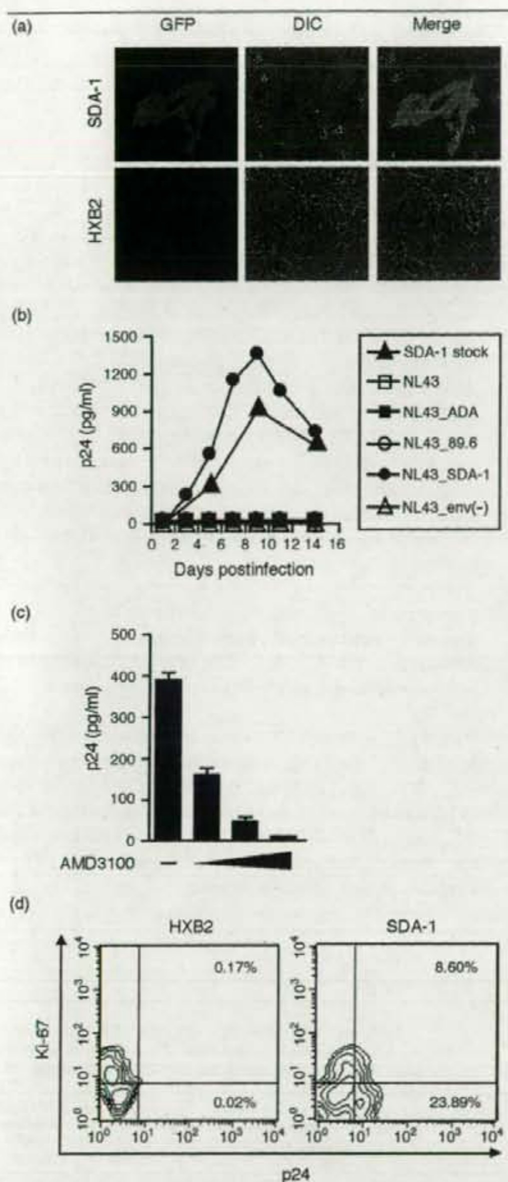

Fig. 4. SDA-1 enters and replicates in CD4$^-$ human p-hepatocytes. (a) Entry of SDA-1 into p-hepatocytes. The p-hepatocytes were exposed to the indicated HIV-1 GFP-p viruses for 48 h. Infectivity was determined as GFP$^+$ cells by confocal microscopy. (b) Replication of SDA-1 Env-chimeric viruses and SDA-1 virus stock in human p-hepatocytes. (c) SDA-1 infects p-hepatocytes through CXCR4. The inhibitory effects of AMD 3100 (0.1, 0.3 and 1.0 μM) on SDA-1 Env-chimeric viruses infection of p-hepatocytes were studied. Results shown are means of triplicate experiments. Bars, SD. (d) SDA-1 replicates in both proliferating and static human p-hepatocytes. Intracellular stainings of HIV-1-infected p-hepatocytes for p24 and Ki-67 were analyzed by flow cytometry. CXCR4, chemokine (C-X-C motif) receptor 4; GFP-p, GFP-pseudotypes.

percentage of p24 expression between Ki-67$^+$ (31%) and Ki-67$^-$ p-hepatocytes (33.1%), suggesting that SDA-1 efficiently enters and replicates in both proliferating and static hepatocytes.

Considering that SDA-1 can infect hepatocytes *in vitro*, it would have been interesting to determine whether the patient's liver was infected *in vivo*. However, consent for a liver biopsy was denied by the patient's family. There was no evidence of liver dysfunction. When virus was isolated from this patient; however, liver damage [an aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio ≥ 1] was observed at the end of the clinical stage. Although the cause of liver injury was unclear, our present data suggest that CD4-independent HIV-1 infection may lead to hepatocellular damage.

Discussion

In this study, we characterized a quasispecies of a CD4-independent HIV-1 isolate, termed SDA-1, which was able to utilize either CXCR4 or CCR5 in the absence of CD4. Moreover, we demonstrated that SDA-1 efficiently entered and replicated in Huh-7 hepatoma cells and normal human hepatocytes, through CXCR4, without inducing apoptotic cell death.

Many SIV and HIV-2 isolates can infect cells without CD4, at least to some extent. However, CD4-independent HIV-1 viruses have been rarely isolated and, so far, only a few laboratory-adapted CD4-independent HIV-1 variants have been reported. It must be noted that CD4-independent HIV-1 variants, isolated *in vitro* by passage through cells lacking CD4, have been shown to be more sensitive to neutralizing antibodies than CD4-dependent viruses [44,45]. Therefore, we might hypothesize that the emergence of a quasispecies of HIV-1 with a reduced requirement for CD4 is likely to be at a low abundance relative to the more common CD4$^+$ strains. However, with disease progression, HIV-1 variants with reduced affinity for CD4 and with increased affinity for chemokine receptor could evolve and become more robust in the viral quasispecies, disseminate in a variety of CD4$^-$ tissues *in vivo* under conditions of both reduced immunological pressure and a dramatically reduced pool of target CD4$^+$ cells concomitant with high levels of virus replication. It will be important to search the viral quasispecies in other patients, especially in the later stages of HIV-1 disease for the existence of similar CD4-independent HIV-1 variants and expanded cellular tropism.

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Although the extent to which CD4⁻ cells are infected *in vivo* is unclear, it has been widely thought to be low. Nonetheless, recent studies [11,12] utilizing the novel approach of laser capture microscopy have revealed HIV-1 sequences in isolated $CD4^-$ cells of kidney epithelium and neuronal cells, indicating that latent infection might occur in such cells or tissues *in vivo*. The mechanism of viral entry into $CD4^-$ cells remains unclear, but as we show here the evidence of emergence of CD4-independent strains *in vivo* must be kept in mind.

End-stage liver disease is now becoming a frequent cause of death in HIV-1-infected hospitalized patients. HCV and HBV coinfection with HIV-1 has been shown to enhance the progression of liver damage [16]. However, little attention has been given to the direct virological interaction between HIV and HCV/HBV in the liver, as HIV has been thought not to infect hepatocytes directly. Nonetheless, a number of reports have documented that histological liver abnormalities occurred solely as a result of HIV-1 infection. In our study, we clearly demonstrated that SDA-1 efficiently enters and replicates in both proliferating and static hepatocytes through CXCR4. To our knowledge, this is the first report that HIV-1 can efficiently replicate in normal hepatocytes. Furthermore, we have shown that HIV-1 infection did not induce significant cytocidal effects in the hepatocytes. It is noteworthy that the liver is a continuously regenerating organ. Therefore, if HIV-1 enters and integrates its DNA into the host genome, liver cells containing HIV-1 DNA will be continuously generated by the division of the infected cells. Thus, the expression of HIV-1 proteins on the infected cell surface might result in chronic damage of the liver cells by inducing host immune responses. Direct virological interaction between HIV, HCV and HBV in the liver or enhanced production of HIV-1 by inflammatory cytokines produced by the HCV and HBV-activated immune cells might also exacerbate the liver injury. At present, however, we have no definite information concerning the extent to which patients' hepatocytes harbor HIV-1 and CD4-independent HIV-1 variants.

Finally, a particularly important area of vaccine research is to take advantage of gp120 structural information to guide the design of novel envelope immunogens. As has been reported, CD4-dependent viruses hide neutralizing epitopes and only CD4 binding to gp120 induces conformational changes in gp120 to fully expose epitopes for broadly neutralizing antibodies. The CD4-independent strain we isolated here seems particularly important, as it can efficiently replicate in $CD4^-$ hepatocytes. Therefore, the gp120 structural alterations, which might expose the coreceptor binding site without binding to CD4, may also open up other sites that could yield neutralizing antibodies. Nevertheless, evidence of a clinical CD4-independent R5X4 HIV-1 virus should have important implications concerning the range of mutability and tropism of HIV-1 and the pathogenesis of AIDS.

Acknowledgements

We would like to thank Dr Shinji Okada for the clinical data of this patient. We also thank to Dr Y. Koyanagi, Dr T. Hara, Dr R. Furuta and H. Sakai for technical supports for isolation of the virus, sequences analysis, cell–cell fusion assay and chimeric viruses of SDA-1, respectively. We are indebted to Dr D.R. Littman for the gift of HIV-1 luciferase and GFP reporter plasmids, Dr C.D. Weiss for Env expression plasmids, and Dr N. Laudau for HOS.CXCR4 and HOS.CCR5 cells. We thank Division of AIDS, NIAID, NIH for providing AMD3100 through the NIH AIDS Research and Reference Reagent Programme. We also thank . Takeda Pharmaceutical Company Ltd. (Osaka, Japan) for providing TAK-779. We are grateful for Dr M. Robert-Guroff for critical reading of the manuscript.

The present work was supported by Grant-in-Aid for Scientific Research B from JSPS and the Scientific Research Expenses for Health and Welfare Programme from the Ministry of Health and Welfare, Japan.

P.X., H.L., Y.S. and T.H. designed the study. P.X., O.U., Y.S., M.Z., Y.A. and H.G. performed the experiments. P.X., O.U, Y.S., H.L. and T.H. analyzed the data. N.S. and H.H. contributed to the coreceptor expressing cell lines. P.X., H.L., Y.S., O.U., N.S., H.H. and T.H. contributed to writing the paper. T.H. contributed to grant application and financial support.

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A formylpeptide receptor, FPRL1, acts as an efficient coreceptor for primary isolates of human immunodeficiency virus

Nobuaki Shimizu*[1], Atsushi Tanaka[1], Takahisa Mori[1,2], Takahiro Ohtsuki[1,2], Aliful Hoque[1], Atsushi Jinno-Oue[1], Chatchawann Apichartpiyakul[3], Shigeru Kusagawa[4], Yutaka Takebe[4] and Hiroo Hoshino[1,2]

Address: [1]Department of Virology and Preventive Medicine, Gunma University Graduate School of Medicine, Showa-machi, Maebashi, Gunma 371-8511, Japan, [2]21st Century COE Program, Gunma University Graduate School of Medicine, Showa-machi, Maebashi, Gunma 371-8511, Japan, [3]Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand and [4]Laboratory of Molecular Virology and Epidemiology, AIDS Research Center, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Email: Nobuaki Shimizu* - gardy@med.gunma-u.ac.jp; Atsushi Tanaka - atsushit@med.gunma-u.ac.jp; Takahisa Mori - morit@med.gunma-u.ac.jp; Takahiro Ohtsuki - totuki@med.gunma-u.ac.jp; Aliful Hoque - aliful@med.gunma-u.acjp; Atsushi Jinno-Oue - ouea@med.gunma-u.ac.jp; Chatchawann Apichartpiyakul - capichar@mail.med.cmu.ac.th; Shigeru Kusagawa - kusagawa@nig.go.jp; Yutaka Takebe - takebe@nih.go.jp; Hiroo Hoshino - hoshino@med.gunma-u.ac.jp

* Corresponding author

Published: 25 June 2008

Retrovirology 2008, **5**:52 doi:10.1186/1742-4690-5-52

Received: 5 March 2008

Accepted: 25 June 2008

This article is available from: http://www.retrovirology.com/content/5/1/52

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Abstract

Background: More than 10 members of seven-transmembrane G protein-coupled receptors (GPCRs) have been shown to work as coreceptors for human immunodeficiency virus type 1 (HIV-1), HIV type 2 (HIV-2), and simian immunodeficiency viruses (SIVs). As a common feature of HIV/SIV coreceptors, tyrosine residues are present with asparagines, aspartic acids or glutamic acids in the amino-terminal extracellular regions (NTRs).

We noticed that a receptor for N-formylpeptides, FPRL1, also contains two tyrosine residues accompanied by glutamic acids in its NTR. It was reported that monocytes expressing CCR5 and FPRL1 in addition to CD4 are activated by treatment with ligands or agonists of FPRL1. Activated monocytes down-modulate CCR5 and become resistant to infection by HIV-1 strains. Thus, FPRL1 plays important roles in protection of monocyptes against HIV-1 infection. However, its own coreceptor activity has not been elucidated yet. In this study, we examined coreceptor activities of FPRL1 for HIV/SIV strains including primary HIV-1 isolates.

Results: A CD4-transduced human glioma cell line, NP-2/CD4, is strictly resistant to HIV/SIV infection. We have reported that when NP-2/CD4 cells are transduced with a GPCR having coreceptor activity, the cells become susceptible to HIV/SIV strains. When NP-2/CD4 cells were transduced with FPRL1, the resultant NP-2/CD4/FPRL1 cells became markedly susceptible to some laboratory-adapted HIV/SIV strains. We found that FPRL1 is also efficiently used as a coreceptor by primary HIV-1 isolates as well as CCR5 or CXCR4.

Amino acid sequences linked to the FPRL1 use could not be detected in the V3 loop of the HIV-1 Env protein. Coreceptor activities of FPRL1 were partially blocked by the forymyl-Met-Leu-Phe (fMLF) peptide.

Conclusion: We conclude that FPRL1 is a novel and efficient coreceptor for HIV/SIV strains. FPRL1 works as a bifunctional factor in HIV-1 infection. Namely, the role of FPRL1 in HIV-1 infection is protective and/or promotive in different conditions. FPRL1 has been reported to be abundantly expressed in the lung, spleen, testis, and neutrophils. We detected mRNA expression of FPRL1 in 293T (embryonal kidney cell line), C8166 (T cell line), HOS (osteosarcoma cell line), Molt4#8 (T cell line), U251MG (astrocytoma cell line), U87/CD4 (CD4-transduced glioma cell line), and peripheral blood lymphocytes. Roles of FPRL1 in HIV-1 infection *in vivo* should be further investigated.

Background

More than 10 members of seven-transmembrane G protein-coupled receptors (GPCRs) support the entry of human immunodeficiency virus type 1 (HIV-1), HIV type 2 (HIV-2), and simian immunodeficiency viruses (SIVs) into target cells as coreceptors in collaboration with the primary receptor CD4 [1,2].

A chemokine receptor (CKR), CXCR4, was firstly shown to work as a coreceptor for HIV-1 strains [3]. Next, a CKR, CCR5, was also identified as a coreceptor for HIV-1 strains [4,5]. Infection of T cells or macrophages with HIV-1 strains that can use CCR5 as a coreceptor (i. e., R5 strains) is an initial event in the establishment of HIV-1 infection *in vivo*, since peoples harboring mutant alleles in the CCR5 gene have been found to be highly resistant to infection by HIV-1, even if they have been estimated to have repeatedly been exposed to the viruses [6,7]. During progression of stages in HIV-1 infection, HIV-1 strains that use CXCR4 as a coreceptor (X4 strains), especially subtype B strains, have been detected [8]. HIV-1 strains that can use both CCR5 and CXCR4 (R5-X4 strains) often emerge, but their roles in pathogenesis of acquired immune deficiency syndrome (AIDS) remain to be elucidated [9]. Thus, these two coreceptors, CCR5 and CXCR4, have been thought to play major roles in HIV-1 infection and the development of related disorders.

Some GPCRs, especially CKRs, play a major role in migration of lymphocytes (chemotaxis) and consequently in the development of inflammation together with their ligands, chemotactic cytokines (chemokines) [10-12]. CKRs are classified into five groups, CC-, CXC-, CX3C-, and XC-CKRs, and other CKRs according to the well conserved amino acid motifs of their ligands [13]. Some CKRs have been shown to act as coreceptors for HIV-1, HIV-2 or SIV (HIV/SIV) strains. In addition to CCR5 and CXCR4, three CC-CKRs, CCR2b, CCR3, CCR8, and D6 have been shown to be alternative coreceptors mainly used by R5 or X4 HIV-1 strains [14-16]. Two CXC-CKRs, CXCR5/BLR1 and CXCR6/BONZO, act as coreceptors for several HIV/SIV strains [17-19]. A CX3C-CKR, CX3CR1/V28, functions as a coreceptor for several HIV-1 strains [20]. In addition to CKRs, several GPCRs, e. g., APJ [21], ChemR23 [22], GPR1 [23], GPR15 [24], RDC1 [25], and the leukotrien B4 receptor LTB4 [26], have been reported to work as coreceptors for HIV/SIV strains. However, the roles of these coreceptors in HIV-1 infection *in vivo* have not been elucidated [27].

We noticed that as a common feature of most CKRs, as well as HIV/SIV coreceptors, tyrosine residues with asparagines, aspartic acids or glutamic acids are present in the amino-terminal extracellular regions (NTRs) [28,29]. A receptor for N-formylpeptides, FPRL1, also contains two tyrosine residues accompanied by glutamic acids in its NTR [30]. FPRL1 has been reported to be expressed in the lung, spleen, and testis, and in neutrophils, and to play an important role in the activation of neutrophils [31]. Monocytes expressing FPRL1 in addition to CD4 are activated by treatment with ligands or agonists of FPRL1. Activated monocytes down-modulate CCR5 and thus become resistant to infection by R5 HIV-1 strains. [32]. In this report, we demonstrate that FPRL1 itself has the capacity to support the entry of various HIV/SIV strains, including primary HIV-1 isolates, into target cells as a novel coreceptor.

Results

FPRL1 as a novel candidate HIV/SIV coreceptor

Major HIV/SIV coreceptors, CCR5 and CXCR4, contain tyrosines and these tyrosines in NTRs of CCR5 and CXCR4 have been demonstrated to be necessary for their coreceptor activities [28,33]. All CKRs reported to have HIV/SIV coreceptor activities harbor tyrosines in their NTRs. Most non-CKR GPCRs that were reported to function as HIV/SIV coreceptors also harbor tyrosines accompanied by aspartic acids, glutamic acids or asparagines in their NTRs (Additional file 1).

Therefore, to discover a novel candidate coreceptors of HIV/SIV, we constructed a phylogenetic tree of peptide receptors for 36 GPCRs containing reported HIV/SIV coreceptors (20 CKRs, and 16 non-CKR GPCRs) using the ClustalW program [72] (Fig. 1). The peptide receptors were clustered into several distinct branches corresponding to the subfamilies of GPCRs. In this phylogenetic tree, we found that CKRs were closely related to each other and

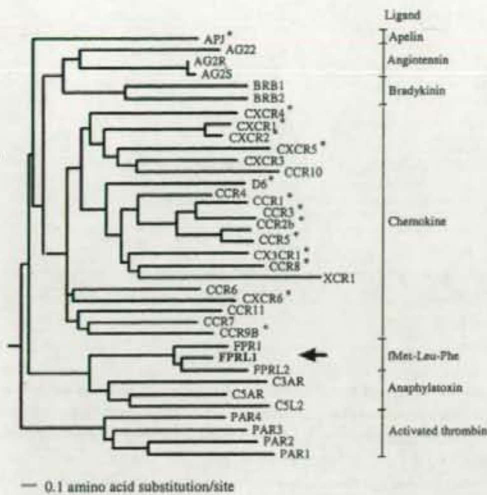

Figure 1

Phylogenetic tree of peptide receptors belonging to the GPCR family. The phylogenetic tree for 20 CKRs and 16 GPCRs related to CKRs was constructed by the ClustalW program [72] according to the methods described in the DDBJ website (National Institute of Genetics, Center for Information Biology and DNA Databank of Japan, http://www.ddbj.nig.ac.jp). FPRL1 is indicated by the arrow. GPCRs reported to function as HIV/SIV coreceptors are indicated by "*".

that all of the formylpeptide receptors reported so far, FPRL1, FPRL2 and FPR1, constituted a distinct subgroup, closely located at positions near CKRs and anaphylatoxin receptors, some of which have been demonstrated to act as coreceptors for HIV/SIV [22]. FPRL1, unlike FPRL2 or FPR1, has tyrosine residues accompanied by asparagines, aspartic acids, and glutamic acids in its NTR (see Additional file 1). Therefore, we focused on a formylpeptide receptor, FPRL1, as a novel candidate coreceptor for HIV/SIV.

Susceptibility of NP-2/CD4/FPRL1 cells to cell line-adapted HIV-1 strains

FPRL1 ORF DNA was amplified using cDNA made from C8166 cell mRNA and cloned into the expression vector pCX-bsr. The expression level of the FPRL1 gene in NP-2/CD4 cells transfected with it, NP-2/CD4/FPRL1, was determined by RT-PCR. mRNA expressions of CCR5, CXCR4, and GPR1 were also detected in NP-2/CD4/CCR5, NP-2/CD4/CXCR4, and NP-2/CD4/GPR1 cells by RT-PCR, respectively. A comparison of the intensity of each PCR band shown in Fig. 2A suggests that the amount of FPRL1 mRNA in NP-2/CD4/FPRL1 cells was 10–100 fold more abundant than the mRNA of CCR5 in NP-2/CD4/CCR5 cells, CXCR4 in NP-2/CD4/CXCR4 cells or GPR1 in NP-2/CD4/GPR1 cells.

To clarify whether FPRL1 has the ability to serve as a coreceptor, the susceptibility of NP-2/CD4/FPRL1 cells to nine cell line-adapted HIV-1 strains was investigated. NP-2/CD4/FPRL1 cells were found to be susceptible to the GUN-1WT, GUN-4V, and GUN-7WT cell-line-adapted HIV-1 strains: approximately 0.5, 5 and 30% of the cells became HIV-1 antigen-positive on day 6 after infection, respectively (Fig. 3A). NP-2/CD4/FPRL1 cells were resistant to infection by IIIB, Ba-L, GUN-1V, GUN-4WT, GUN-7V, and SF162 strains: less than 0.1% cells were HIV-1 antigen-positive on day 6 after infection. NP-2/CD4 cells, in which no expression of the FPRL1, CCR5, CXCR4, or GPR1 gene was detected by RT-PCR (Fig. 2B), were completely resistant to infection by all HIV-1 strains tested (Fig. 3E), as previously described [49]. Thus, FPRL1 enabled infection of several cell line-adapted HIV-1 strains as a coreceptor.

As controls, the susceptibilities of NP-2/CD4/CXCR4, NP-2/CD4/CCR5, and NP-2/CD4/GPR1 cells to HIV-1 strains were also examined. NP-2/CD4/CXCR4 cells were highly susceptible to all HIV-1 strains, except the Ba-L and SF162 strains, when tested on day 6 after infection (Fig. 3B), while NP-2/CD4/CCR5 cells were highly susceptible to five HIV-1 strains, Ba-L, GUN-1WT, GUN-4WT, GUN-7WT, and SF162, but not to the IIIB strain (Fig. 3C). NP-2/CD4/GPR1 cells were susceptible to three HIV-1 variants, GUN-1V, GUN-4V, and GUN-7V, but not to three HIV-1 strains, IIIB, Ba-L, and SF162 (Fig. 3D). The coreceptor uses of the cell line-adapted HIV-1 strains are summarized (see Additional file 2) and as follows: IIIB (coreceptor use, X4), Ba-L (R5), GUN-1WT (FPRL1-R5-X4), GUN-1V (GPR1-X4), GUN-4WT (R5-X4), GUN-4V (FPRL1-GPR1-X4), GUN-7WT (FPRL1-R5-X4), GUN-7V (GPR1-X4), and SF162 (R5). We have reported that there are one or two amino acid mutations in the V3 region of gp120 between GUN-1WT and GUN-1V, between GUN-4WT and GUN-4V, and between GUN-7WT and GUN-7V [59]. Our results suggest that amino acid sequences of the V3 region markedly affected FPRL1 use as a coreceptor by HIV-1 strains.

FPRL1 as a coreceptor for primary isolates of HIV-1

Next, we investigated whether FPRL1 also acts as a coreceptor for primary HIV-1 isolates. HIV-1 strains, AG204, AG206, AG208, HCM303, HCM305, HCM308, HCM309, HCM342, mIDU101, and mSTD104, were isolated from PBLs derived from HIV-1-infected Vietnamese or Myanmanese subjects and had been propagated only in PBLs before this experiment.

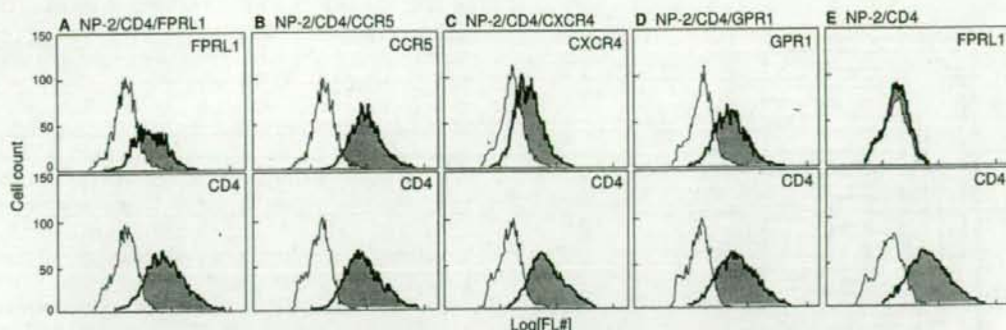

Figure 2
Expression of GPCR mRNA in various types of human cells detected by RT-PCR. (A) Relative amount of mRNA expression for CCR5, CXCR4, FPRL1 and GPR1 in NP-2/CD4 cells expressing the coreceptors. RT-PCR was done using serially diluted (1:1, 1:10, 1:100, 1:1000, and 1:10000) cDNA reverse-transcribed from the total RNA. As a control, the expression level of GAPDH mRNA in each cDNA preparation was determined by RT-PCR. (B) mRNA expression for four GPCRs in 11 kinds of human cells as detected by RT-PCR using the specific primers. As a control, the expression level of GAPDH mRNA in each cDNA preparation was determined by RT-PCR. The PCR primers amplify 1,377 (CD4), 1,059 (CCR5 and CXCR4), 1,056 (FPRL1), 1,068 (GPR1), and 1,008 (GAPDH) base-pair DNA fragments when these genes are expressed in the cells. Expression level, (-~++) were determined by intensities of amplified DNA bands compared to those of the corresponding controls (GAPDH).

When NP-2/CD4/FPRL1 cells were exposed to these isolates, the cells were found to be clearly susceptible to AG204, AG206, HCM308, HCM342, and mSTD104 isolates: 20, 30, 15, 40, and 60% cells, respectively, became HIV-1 antigen-positive by IFA on day 6 after infection and syncytia were formed (Figs. 4A and 5). A large number of syncytia were formed in the infection of NP-2/CD4/FPRL1 cells with the AG206, HCM342, and mSTD104 isolates, suggesting that replication of HIV-1 efficiently occurred in these cells (data not shown). NP-2/CD4/FPRL1 cells also demonstrated lower, but clear susceptibilities to other isolates, AG208, HCM305, and HCM309. NP-2/CD4/FPRL1 cells were not susceptible to HCM303 and mIDU101 isolates. Thus, eight out of the ten primary HIV-1 isolates could infect NP-2/CD4/FPRL1 cells.

NP-2/CD4/CCR5 cells were highly susceptible to AG204, AG206, HCM308, HCM342, mIDU101, and mSTD104 isolates (Fig. 4B), and slightly susceptible to AG208, HCM303, HCM305, and HCM309 isolates.

NP-2/CD4/CXCR4 cells showed a high susceptibility to AG204, AG206, AG208, HCM303, HC305, HCM308, and HCM309 isolate (Fig. 4C), while less than 1% of HIV-1 antigen-positive cells were detected after infection with HCM342, mIDU101, or mSTD104 isolates. NP-2/CD4/GPR1 and NP-2/CD4 cells were resistant to infection by all of these primary isolates (Figs. 4D, 4E, and 5).

The phenotypes of the coreceptor uses of the primary HIV-1 isolates were as follows (see Additional file 2): FPRL1-R5-X4 use, AG204, AG206, AG208, HCM305, and HCM308; FPRL1-R5 use, HCM342 and mSTD104; FPRL1-X4 use, HCM309; R5 use, mIDU101, and X4 use, HCM303. Thus, FPRL1 can work as a coreceptor not only for cell line-adapted HIV-1 strains but also for primary HIV-1 isolates.

Amino acid sequences of the V3 domain of the HIV/SIV Env protein

Cell line-adapted HIV-1 strains, GUN-4V (GPR1-X4) and GUN-7WT (R5-X4), could not use FPRL1 as a coreceptor, whereas their related strains, GUN-4WT and GUN-7V, with one or two amino acid substitutions in the V3 region, could use FPRL1 (Fig. 3 and see Additional file 2). This finding raised the possibility that a determinant of the FPRL1 use of HIV-1 as a coreceptor lies in the V3 region. Therefore, we determined the amino acid sequences of the V3 regions of primary HIV-1 strains propagated in NP-2/CD4 cells expressing one of the coreceptors. DNA regions coding for the V3 domain of gp120 were amplified by PCR using cellular DNA of NP-2/CD4/FPRL1 cells infected with AG204, AG206, HCM305, HCM309, HCM342, or mSTD104 strains and NP-2/CD4/CXCR4 cells infected with the HCM303 strain as templates. These amplified DNAs were cloned into the TA-cloning vector pTarget and their nucleotide sequences were determined.

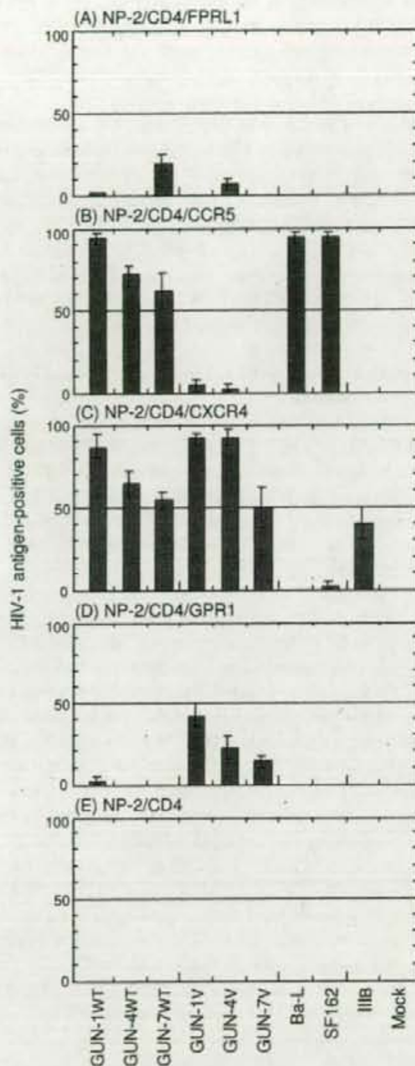

Figure 3
Use of FPRL1, CCR5, CXCR4 or GPR1 as a coreceptor by various cell line-adapted HIV-1 strains. Cells were inoculated with nine HIV-1 strains. The susceptibilities of the cells were determined by IFA six days after viral inoculation. The coreceptor uses of these HIV-1 strains are summarized (see Additional file 2). NP-2/CD4 cells were also tested up to eight days after inoculation and were completely resistant to all the HIV-1 strains examined.

When their nucleotide sequences were compared with those of HIV-1 isolates submitted to the Genbank and reported previously [34], several nucleotide substitutions were observed in the V3 sequences of the primary isolates infecting NP-2/CD4/CCR5, NP-2/CD4/CXCR4, or NP-2/CD4/FPRL1 cells (data not shown).

No amino acid substitution, however, was detected in the V3 domains, because all the nucleotide substitutions detected in the V3 domains were synonymous (see Additional file 3). Single-amino acid substitutions were detected in the C3 domain of AG206, HCM303, and HCM342 strains propagated in FPRL1-expressing cells. These findings indicate that subtype C or AE HIV-1 strains propagated in PBLs and those propagated in NP-2/CD4 cells expressing CCR5, CXCR4 or FPRL1 have the identical amino acid sequences in the V3 domains. Nevertheless, five HIV-1 strains using FPRL1, GUN-1WT, GUN-7WT, HCM305, HCM309, and HCM342, had threonine at the 13th amino acid position of the V3 region, while two HIV-1 strains which did not use FPRL1 as a coreceptor, GUN-4WT and HCM303, had serine at this position. The amino acids at this position may be responsible for determining FPRL1 use by these HIV-1 strains.

HIV-1 samples produced by NP-2/CD4/FPRL1 cells that had been infected with the AG204, AG206, HCM308, and HCM342 strains could use CCR5 as a coreceptor (data not shown). These results suggest that the primary HIV-1 samples are not a mixture of FPRL1-tropic virus and R5-tropic virus, and that HIV-1 isolates using FPRL1 can also use CCR5 as a coreceptor.

FPRL1 as a coreceptor for HIV-2 and SIV strains

Next, we tested a coreceptor activity of FPRL1 for four HIV-2 and SIV strains. NP-2/CD4/FPRL1 cells were highly susceptible to two HIV-2 strains CBL23 and ROD/B: about 60% of cells became HIV-2 antigen-positive on day 6 after infection (Fig. 6A). As for the GH-1 and SBL6669 HIV-2 strains, and mndGB-1 SIV strain, 30, 15, and 30% of the cells, respectively, were infected. NP-2/CD4/FPRL1 cells were, however, resistant to the R5 SIV strain mac251.

As reported previously [49], NP-2/CD4/CCR5 cells were susceptible to four HIV-2 strains (CBL23, GH-1, ROD/B, and SBL6669) and two SIV strains (mac251 and mndGB-1) (Fig. 6B). NP-2/CD4/CXCR4 and NP-2/CD4/GPR1 cells were susceptible to these four HIV-2 strains and the mndGB-1 strain (Figs. 6C and 6D), but not to the mac251 strain. Thus, the coreceptor uses of HIV-2 and SIV strains are summarized (see Additional file 2). FPRL1 may work as a coreceptor for HIV-2 and SIV strains with an R5-X4-GPR1 phenotype, but not those with an R5 phenotype.

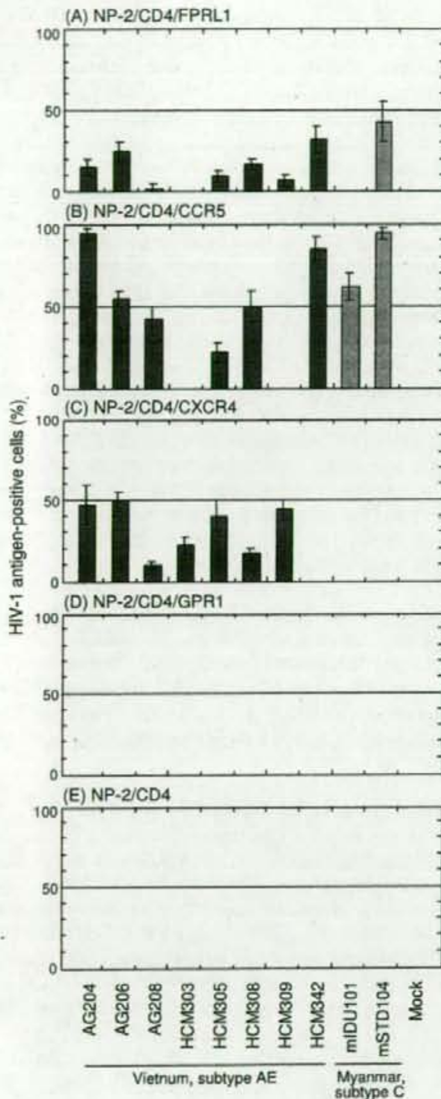

Figure 4

Use of FPRL1, CCR5, CXCR4 or GPR1 as a coreceptor by various primary HIV-1 isolates. The susceptibilities of cells to HIV-1 isolates were determined by IFA six days after viral inoculation. NP-2/CD4 cells were completely resistant to all these HIV-1 isolates (E). The origins and subtypes of these primary isolates are summarized (see Additional file 2).

CD4 dependency of the coreceptor activity of FPRL1

Some HIV-2 strains have been shown to enter CD4-negative cells, and this entry is mediated through coreceptors [35,36]. To clarify whether infection of HIV/SIV mediated through FPRL1 is dependent on CD4 or not, NP-2/CD4/FPRL1 and NP-2/CD4/CCR5 cells were pretreated with serially-diluted anti-CD4 MoAb, NuTH/I, before inoculation. Relative percentages of infected cells are shown in Fig. 7A. NuTH/I MoAb (10 μg/ml) almost completely inhibited infection of NP-2/CD4/FPRL1 cells, as well as NP-2/CD4/CCR5 cells, with all HIV/SIV strains tested, *i. e.*, GUN-7WT, HCM342, CBL23, and mndGB-1, suggesting that FPRL1 mediates infection of HIV/SIV as a coreceptor, *i. e.*, in a CD4-dependent manner.

Partial inhibition of the coreceptor activity of FPRL1 by the fMLF peptide

It has been reported that the coreceptor functions of CCR5 and CXCR4 for HIV-1 infection can be inhibited by their ligands, RANTES and SDF-1β, respectively [37,38]. Some ligands have been used as starting materials to find and develop anti-HIV reagents. We examined the inhibitory effects of an FPRL1 ligand, fMLF peptide, on infection with HIV/SIV.

NP-2/CD4/FPRL1 cells were pretreated with the fMLF peptide (100 μg/ml). As a control, NP-2/CD4/CCR5 cell were also pretreated with a chemokine, RANTES, a ligand for CCR5. As shown in Fig. 7B, the fMLF peptide (100 μg/) showed a partial inhibitory effect on infection of NP-2/CD4/FPRL1 cells with GUN-7WT, HCM342, CBL23, or the mndGB-1 strain. Infection of NP-2/CD4/CCR5 cells with CBL23 and mndGB-1, but not with the GUN-7WT or HCM342 strains, was also partially blocked by the peptide, suggesting that the fMLF peptide may have inhibitory effects on infection of CCR5-positive cells by several strains of HIV/SIV. The difference in inhibitory effects of the fMLF peptide may reflect the HIV/SIV strain-dependent interaction with CCR5. On the contrary, as shown in Fig. 7C, a chemokine, RATES, had hardly any effect on infection of NP-2/CD4/FPRL1 cells with the HIV/SIV strain. RANTES blocked infection of NP-2/CD4/CCR5 cells with HIV/SIV strains by 50–80%, as reported [37].

Expression of FPRL1 mRNA in a wide variety of cells

We investigated the expression of FPRL1, CCR5, CXCR4, and GPR1 mRNA in cells originating from various types of human tissues by RT-PCR. CD4 and GAPDH mRNA were detected as controls. Fig. 2B shows that FPRL1 mRNA was detected abundantly in C8166 (T cell line), Molt4#8 (T cell line), U251MG (astrocytoma), and 293T (embryonal kidney) cells. Faint signals of FPRL1 mRNA were detected in HOS (osteosarcoma), U87/CD4 (CD4-transduced glioma cell line) cells, and PBLs. The expression levels of FPRL1 mRNA in Molt4#8, U251MG, and 293T cells were

Figure 5
The susceptibilities of NP-2/CD4/GPCR cells to HIV-1 strains. Cells were infected with two primary HIV-1 isolates, AG204 and mSTD104, and a cell line-adapted strain, Ba-L. Six days after infection, cells positive for HIV-1 antigens were detected by IFA using a fluorescence microscope. Percentage of cells judged to be positive for IFA are shown.

estimated to be comparable to those of CXCR4 in these cells. FPRL1 mRNA was, however, not detected in HepG2 (hepatoblastoma), Huh7 (hepatoma), or NP-2/CD4 (CD4-transduced glioma) cells. CCR5 mRNA was detected in Molt4#8, U251MG, and 293T cells, even though the levels were much lower than those of FPRL1 mRNA. CXCR4 mRNA was detected clearly in C8166 and Molt4#8 cells, and weakly in 293T, HepG2, HOS, Huh7, U251MG cells, and PBLs. CD4 mRNA was detected in BT-20N, C8166, Molt#3, NP-2/CD4, U87/CD4 cells, and PBLs. Similar amounts of GAPDH mRNA were detected in all cells tested here. No signal was seen in RNA samples without reverse transcriptase treatment, indicating that the chromosomal DNA did not contaminate the cDNA preparations. FPRL1 are expressed abundantly in various types of cells derived from not only lymphoid tissues, but also the brain.

Discussion

The genomic diversity of HIV-1 is thought to be generated by the low fidelity of its reverse transcription and frequent recombination of the genome [39]. Mutation of amino acid sequences in the V3 domain of the Env can give HIV-1 the ability to use various GPCRs as coreceptors. Coreceptors other than CCR5 and CXCR4, that are related to the clinical involvement of the HIV-1 infection have not yet been clarified. We planned to identify novel coreceptors that can be frequently used not only by cell line-adapted HIV-1 strains, but also by primary HIV-1 isolates. In this study, we focused on a formylpeptide receptor, FPRL1, which is located genetically close to CKRs in the phylogenetic tree we made, containing three tyrosines in its NTR (Fig. 1 and see Additional file 1) as a candidate for a novel coreceptor.

First, we examined the coreceptor activity of FPRL1 for cell line-adapted HIV/SIV strains. We found that FPRL1 worked as a coreceptor for several HIV-1 strains, GUN-4V (coreceptor use: FPRL1-X4-GPR1) and GUN-7WT (FPRL1-R5-X4), but not GUN-1WT (R5-X4), GUN-1V (X4-GPR1), GUN-4WT (R5-X4), GUN-7V (X4-GPR1), IIIB (X4), Ba-L (R5), or SF162 (R5) (Fig. 3). Thus, FPRL1 use by cell line-adapted HIV-1 strains does not correlate with their use of CCR5, CXCR4 or GPR1. We showed that the R5-X4 phenotype of the GUN-4WT and GUN-7WT strains can be changed to the X4-GPR1 phenotype of GUN-4V and GUN-7V variants by one or two amino acid substitutions at the V3 loop [59]. Therefore, the V3 loop is thought to be a determinant of FPRL1 use, as well as GPR1 use, by HIV-1. Like other coreceptors for HIV-2 and SIV strains, FPRL1 was also efficiently used by R5-X4-dual-tropic HIV-2 and SIV strains (Fig. 6).

Next, to examine the possibility that FPRL1 is involved in HIV-1 infection *in vivo*, we examined the susceptibility of NP-2/CD4/FPRL1 cells to primary HIV-1 isolates. It is generally thought that coreceptors other than CCR5 or CXCR4 are little used by primary HIV-1 isolates *in vivo* [40]. We found, however, that FPRL1 could work as a coreceptor for many primary HIV-1 isolates of subtype AE or C (Figs. 4 and 5). NP-2/CD4/FPRL1 cells were susceptible not only R5-X4 HIV-1 isolates (AG204, AG206, AG208, HCM305, and HCM308), but also R5 HIV-1 isolates (HCM342, and mSTD104) or an X4 HIV-1 isolate (HCM309) (see Additional file 2). NP-2/CD4/FPRL1 cells were not susceptible to R5-tropic HCM303 and X4-tropic mIDU101 isolates. Thus, we concluded that the FPRL1 use by HIV-1 strains does not coincide with the use of CCR5, CXCR4 or GPR1.

The ratio of FPRL1 use was as high as 80% for the primary HIV-1 isolates belonging to subtypes AE and C. This ratio for subtype B primary HIV-1 isolates was, however, much lower according to our preliminary examination (data not shown), suggesting that FPRL1 use may be linked to infection with subtypes AE and C HIV-1.

It is intriguing that only a few cell line-adapted HIV-1 strains could use FPRL1 as a coreceptor, whereas many primary HIV-1 isolates propagated in PBLs could do so. It is probable that a population of HIV-1 that uses FPRL1 as a coreceptor in addition to CCR5 or CXCR4 may have

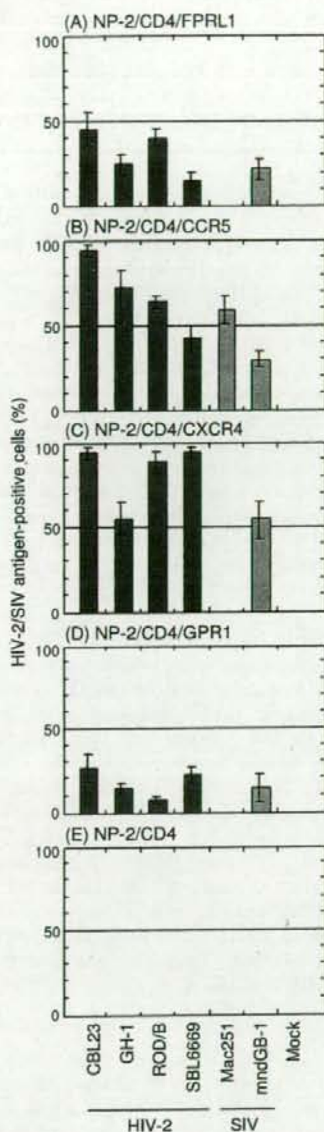

Figure 6
Use of FPRL1, CCR5, CXCR4 or GPR1 as a coreceptor by HIV-2 and SIV strains. The susceptibilities of cells to four HIV-2 strains and two SIV strains were determined by IFA six days after viral inoculation. NP-2/CD4 cells were completely resistant to these HIV-2 strains (E). These results are summarized (see Additional file 2).

been lost in the course of propagation of HIV-1 strains *in vitro* using cell lines because it was markedly smaller than the HIV-1 population which did not use FPRL1, but did use CCR5 or CXCR4.

It has been shown that determinants for CCR5, CXCR4, and GPR1 uses by HIV-1 strains lie in the V3 domain of the Env protein [53,41]. Amino acid substitutions that are apparently linked to FPRL1 use could be identified in the V3 domain of GUN-1WT, GUN-4V, and GUN-7WT strains. That is, the amino acid substitutions at the tip of the V3 domain from proline to serine or from proline to threonine can affect FPRL1 use by HIV-1. Therefore, we examined the possibility that any specific amino acid substitution of the Env protein may give HIV-1 the ability to use FPRL1 as a coreceptor in addition to CCR5 or CXCR4 use. Primary isolates, AG204, AG206, AG208, HCM305, HCM308, HCM342, and mSTD104, were inoculated to, and propagated in, NP-2/CD4/FPRL1 cells in addition to NP-2/CD4/CCR5 or NP-2/CD4/CXCR4 cells. Then, HIV-1 DNA in these cells was subjected to DNA sequencing.

A few nucleotide substitutions were detected in the the V3 domain of the *env* gene between HIV-1 propagated in NP-2/CD4/FPRL1 cells and NP-2/CD4/CCR5 or NP-2/CD4/CXCR4 cells (see Additional file 3). The deduced amino acid sequences of the V3 domain of HIV-1 isolates propagating in NP-2/CD4/FPRL1 cells were identical to those propagated in NP-2/CD4/CCR5 or NP-2/CD4/CXCR4 cells. Furthermore, HIV-1 produced by NP-2/CD4/FPRL1 cells that had been infected with the HIV-1 isolates could infect NP-2/CD4/CCR5 or NP-2/CD4/CXCR4 cells (data not shown). Therefore, these primary isolates could use FPRL1 as a coreceptor in addition to CCR5 and/or CXCR4. It is possible that the determinants of FPRL1 use can not be separated from CCR5, CXCR4 or GPR1 use in HIV-1 strains. There is still another possibility that amino acid mutations in regions other than the V3 domain give HIV-1 strains the ability to use FPRL1 as a coreceptor.

fMLF peptides are bacterial products that have potent chemotactic activities for phagocytes. It was reported that FPRL1 activated by the fMLF peptide or peptides derived from the Env glycoprotein gp120 of HIV-1 interferes with the coreceptor function of CCR5 and CXCR4 by down-regulating them, and as a result, these peptides prevent HIV-1 infection [32,42,43]. Desensitization or down-regulation of CCR5 by the fMLF peptide has also been observed in human immature dendritic cells, on which both FPRL1 and CCR5 are expressed [42].

In this study, a partially inhibitory effect of the fMLF peptide on the FPRL1-mediated infection with HIV/SIV strains was observed, while anti-CD4 MoAb NuTH/I could almost completely block it (Figs. 7A and 7B). The