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The NP-2 Cell System for Analyses of the Coreceptor Usage of Human and Simian Immunodeficiency Viruses

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Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) are the causative agents of acquired immune deficiency syndrome. Our interests have focused on HIV entry into target cells and HIV cell tropism. The cell tropism of HIV and the determinants of a cell's susceptibility to HIV infection have been mainly explained by the combination of HIV surface proteins, Env, and expression of CD4 and coreceptors on the target cells. The coreceptors are molecules belonging to the G protein-coupled 7-transmembrane receptors, especially chemokine receptors. We propose that many researchers working in this field have noticed that our system of using a human glioma cell line, NP-2, has been quite useful for the identification of HIV coreceptors, for the determination of coreceptor usage in HIV strains and for the isolation of primary HIV-1 strains, and for the titration of infectivity. The properties of the assay systems using NP-2 cells that we have developed are summarized in this review through an introduction of some of our work. (Kitakanto Med J 2012；62： 1～14)

Key words： HIV-1, CD4 receptor, coreceptor use, AIDS

Introduction

Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) are retroviruses and are the causative agents of acquired immune deficiency syndrome (AIDS). Retroviruses are grouped into three subfamilies: oncovirinae, lentivirinae (lentivirus) and spumavirinae. The HIVs and simian immunodeficiency viruses (SIVs) are members of the lentivirus subfamily. Lentiviruses are highly mutable, similar to influenza virus or hepatitis C virus, and thus the development of effective vaccines against lentiviruses has not yet been achieved.

HIV-1 was isolated in 1983 by a French research group.[1] As early as 1984, CD4 was identified as a factor necessary for HIV-1 entry, namely, as a receptor for HIV-1.[2,3] It was soon realized that not all human cells positive for CD4 were susceptible to HIV-1 infection,[4] and another factor or secondary factor was deemed to be necessary for HIV-1 entry into target cells in addition to CD4 (Fig. 1A and 1B). After 12 years of intense competition to identify a second receptor, it was reported in 1996 that a CXC chemokine receptor, CXCR4, could confer cells positive for CD4 but resistant to HIV-1 infection with the capacity to be infected by T-cell-line-tropic HIV-1 (HIV-1 strains preferentially grow in most T-cell lines).[5] Then, a CC chemokine receptor, CCR5, was also found to be an indispensable factor for the entry of macrophage-tropic HIV-1 strains. Several papers that reported the chemokine receptors as HIV-1 coreceptors were ranked at or near the top of the list of the most cited papers of 1996.[5-8] HIV-1, using CCR5 as a coreceptor (i. e., the CCR5-tropic virus), plays a critical role in the establishment of HIV-1 infection in humans as described below.

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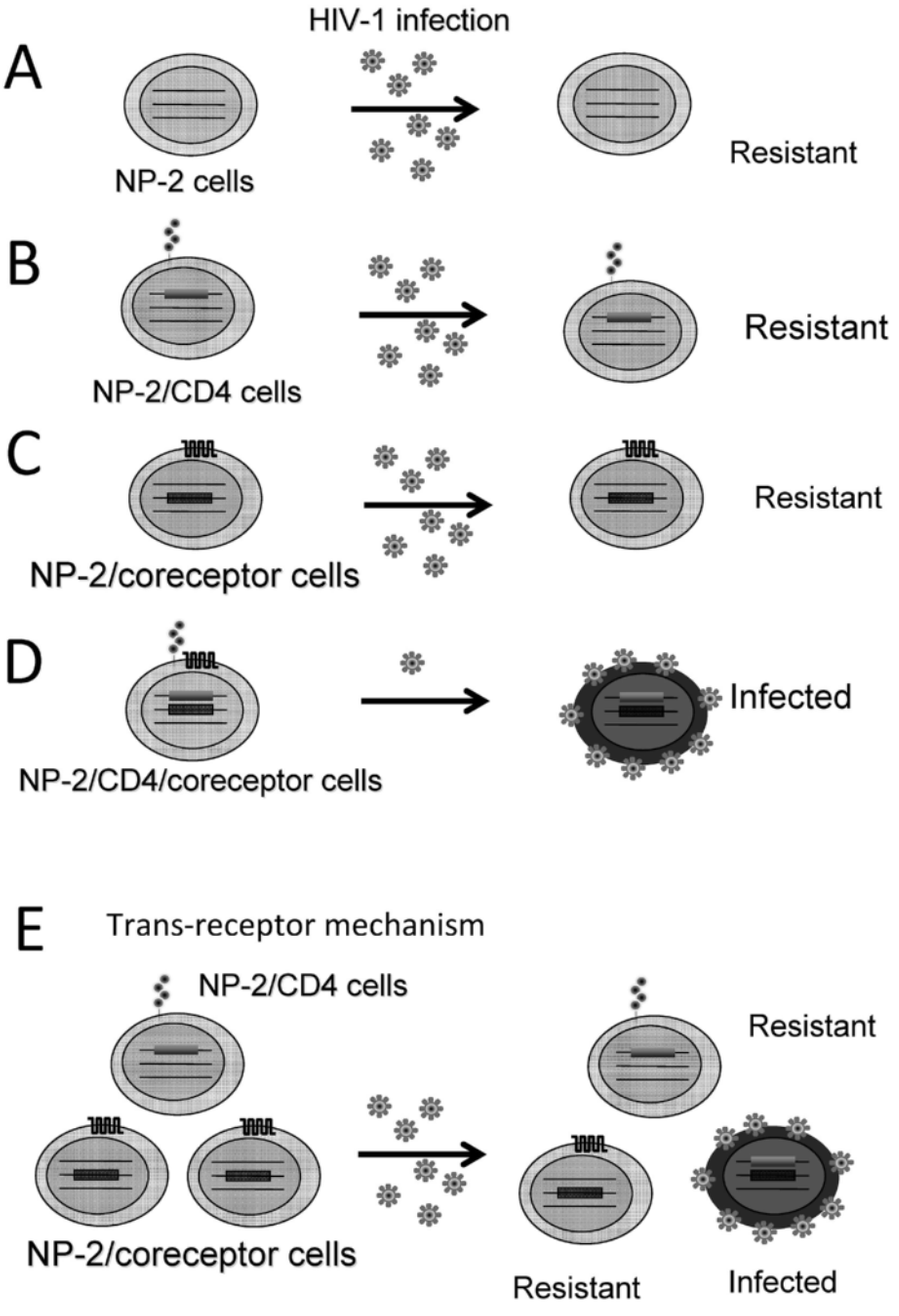

Fig. 1 NP-2, NP-2/CD4 and NP-2/coreceptor cells are resistant to HIV-1 infection while NP-2/CD4/coreceptor cells are highly susceptible to infection with coreceptor-compatible HIV-1 strains (A, B, C and D). According to the trans-receptor mechanism, after infection of a mixture of NP-2/CD4 and NP-2/coreceptor cells with HIV-1, a few NP-2/coreceptor cells are expected to be infected with HIV-1 (E).

Viral and genomic structures of HIV-1

The size of HIV-1 is approximately 100 nm in diameter. There are three major structural protein genes, *gag* (p17, p24 and p15/p7), *pol* (RT : reverse transcriptase and protease), and *env*, encoded by its RNA genome. The envelope (Env) proteins gp120 and gp41 are localized on and throughout the viral membrane, which is mainly comprised of a lipid bilayer derived from host cells[9] (Fig. 2).

Upon HIV-1 entry into cells, the CD4-binding region, C4 of gp120, binds to domain 1 of CD4, which leads to a conformational change of gp120 and permits the V3 region or loop of the Env protein to interact with its coreceptor, CCR5 or CXCR4. This binding results in dislocation of the viral transmembrane protein gp41, and the viral particles (virions) will be connected to the cellular membrane through the fusion domain of gp41. This fusion will lead to further membrane fusion between virions and cells, and viral

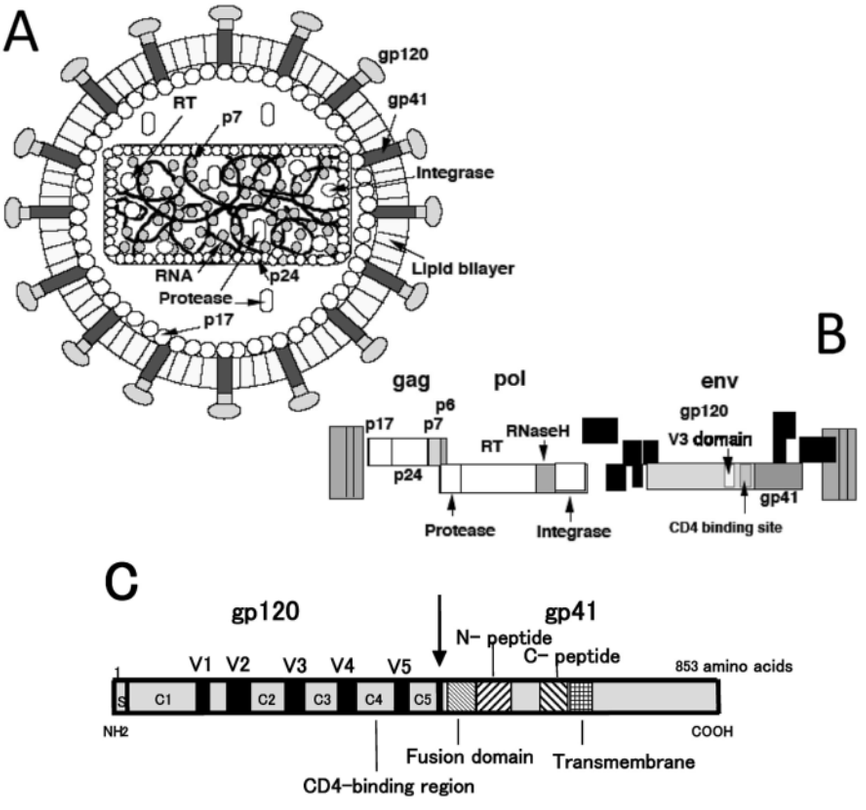

Fig. 2 Structure of HIV-1.
(A) Schematic presentation of HIV-1 virion.
(B) Genetic structure of HIV-1 genome.
(C) Functional regions of HIV-1 Env proteins, gp120 and gp41.

cores harboring HIV-1 genomic RNA enters into the cytoplasm of target cells,[9-11] (Fig. 3). Then, viral RNA is reverse transcribed, and the resultant viral DNA integrates into cellular genome, and thus, the early steps of HIV-1 infection are completed. Then, the late steps, namely, the transcription of viral RNA and synthesis of viral proteins, begin.

Importance of CCR5 in the establishment of HIV-1 infection in vivo

CXCR4 and CCR5 belong to the G protein-coupled 7-transmembrane receptor (GPCR) family (Fig. 4A). In the asymptomatic phases of HIV-1 infection, CCR5-tropic (R5) viruses play a pivotal role in the establishment and persistence of HIV-1 infection in vivo. During the disease progression to symptomatic phases such as AIDS, the shift in the coreceptor use of HIV-1 frequently takes place in subjects infected with subtype B HIV-1. Subtype B is a major subtype of HIV-1 prevalent in developed countries like Japan or USA. CXCR4-tropic or dual-tropic (CCR5- and CXCR4- tropic) (R5X4) variants or HIV-1 strains using co- receptors other than CCR5 and CXCR4 are often detected in patients with early and late stages of immunodeficiency.[12-16]

Some people have been found to be highly resistant to HIV-1 infection, despite highly risky behaviors associated with contracting HIV-1, or to be relatively refractory to disease progression or to AIDS-related syndromes after infection. Molecular epidemiological studies on some of these people revealed that some individuals have a 32-base-pair deletion in the second extracellular loop of *CCR5* gene. This deletion induces a premature termination in the second extracellular loop during translation of CCR5 protein (Fig. 4B).

Those homozygous for this allele are known to be highly resistant to HIV-1 infection. Approximately 1% of Caucasians are heterozygous for this CCR5-delta-32 (CCR5Δ32) allele, and the peripheral blood lymphocytes (PBMCs) of subjects homozygous at this allele are highly resistant to infection by CCR5-tropic but not CXCR4-tropic HIV-1, even in tissue culture experiments. These findings indicate that CCR5 is required for the establishment of HIV-1 infection in humans.[17-20]

The lack of CCR5 may not appreciably influence immunological functions of humans, and thus, CCR5 has been thought to be a good target for the development of new types of anti-HIV-1 agents that will block HIV-1 entry. Such an anti-CCR5 drug, Maraviroc, was approved in Japan in 2008. For the clinical use

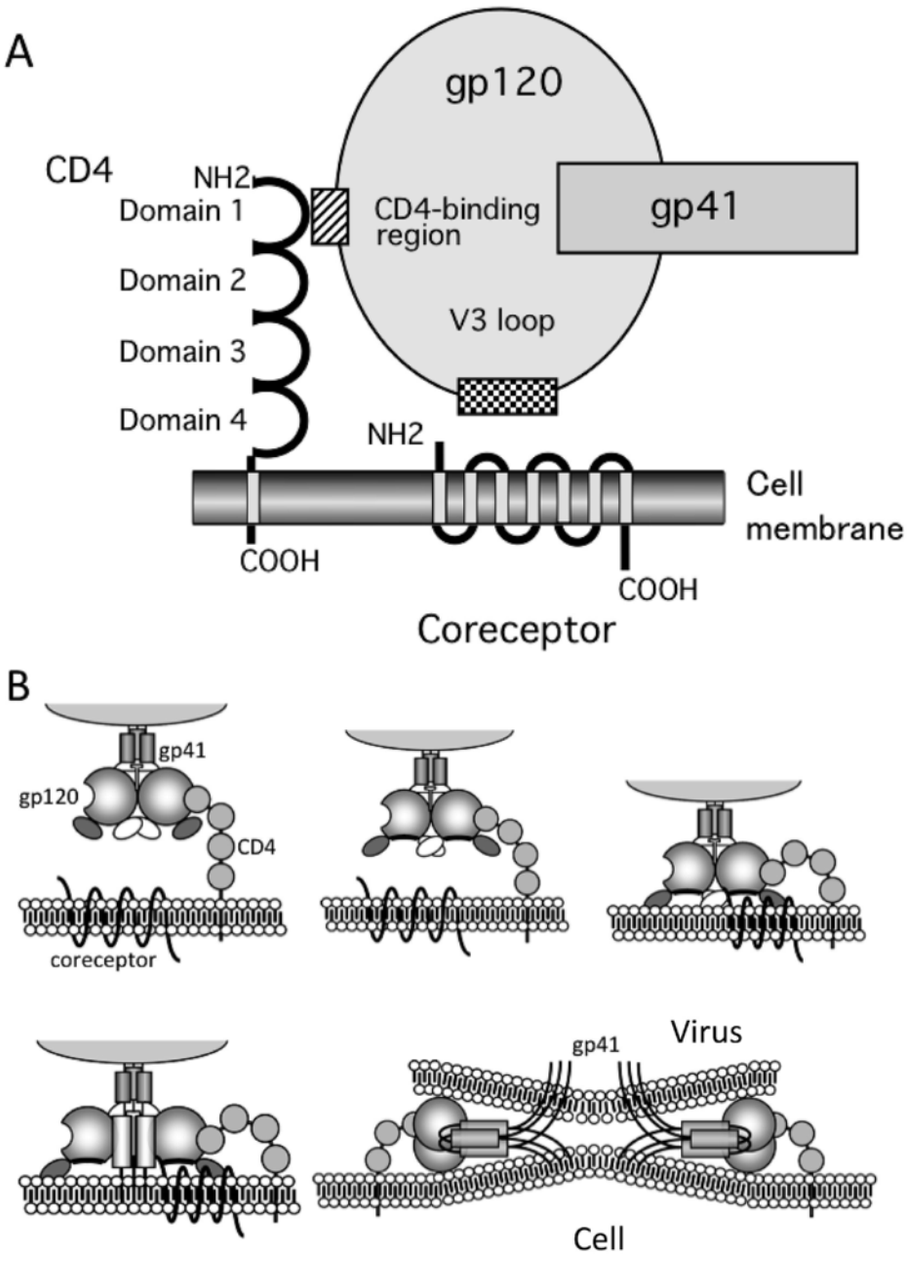

Fig. 3 Roles of CD4 and a coreceptor in HIV-1 infection.
(A) Interaction of the CD4-binding region and V3 loop of HIV-1 gp120 with CD4 and a coreceptor, respectively, of HIV-1-susceptible cells.
(B) Schematic illustlation of fusion processes between virus membrane and cellular membrane.

of this drug, an easy, rapid and accurate [TL2] assay system is necessary for determining the co-receptor use of HIV-1 strain, as the administration of this drug should be limited to subjects that are solely infected with CCR5-tropic HIV-1.[21] The NP-2 cell system described below will be useful for this determination.

GUN strains of HIV-1

We cocultivated human T-cell leukemia virus type 1 (HTLV-1)-positive MT-4, C8166 or ATL-3I T cell lines[22,23] with PBMCs of HIV-1 infected subjects. HTLV-1-positive T cells are known to be highly sensitive to HIV-1 as they express abundant quantities of CD4 and CXCR4 but not CCR5. From these cells, we isolated many HIV-1 strains and named them GUN viruses after Gunma,[24,25] e.g., GUN-1, GUN-2, GUN-3, GUN-4, GUN-5, GUN-6 and GUN-7.

GUN-6 was the first HIV-1 isolate from a foreign patient diagnosed in our University Hospital. Dr. H. Tanami, then a professor at the Central Laboratory in the Hospital, supplied us with this blood sample. We cocultivated the PBMCs from this sample with HTLV-1-positve MT-4 and ATL-3I cells and soon noticed that these T cells were positive for HIV-1 antigens. This viral strain was named GUN-6. The culture supernatant of these T cells was inoculated onto U937

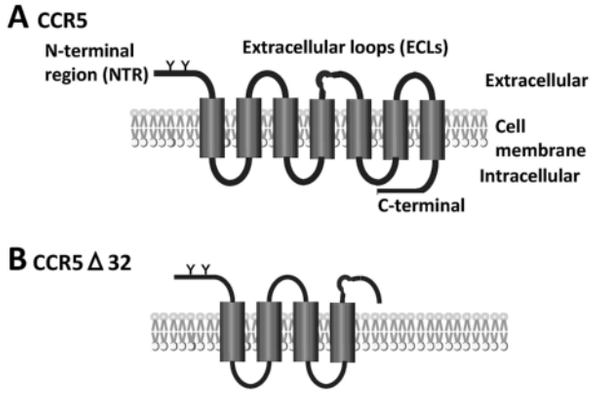

Fig. 4 Schematic presentation of chemokine receptor CCR5. Structures of CCR5 (A) and CCR5Δ32 mutant with 32 base-pair deletion in the genome of the second extracellular loop (B). This deletion leads to premature termination of CCR5 at the second extracellular loop.

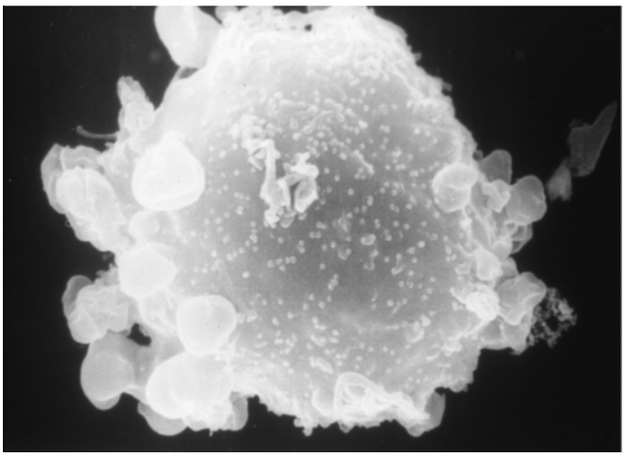

Fig. 5 Scanning electron micrograph of a human monocytic cell, U937, persistently infected with an HIV-1 strain, GUN-6.

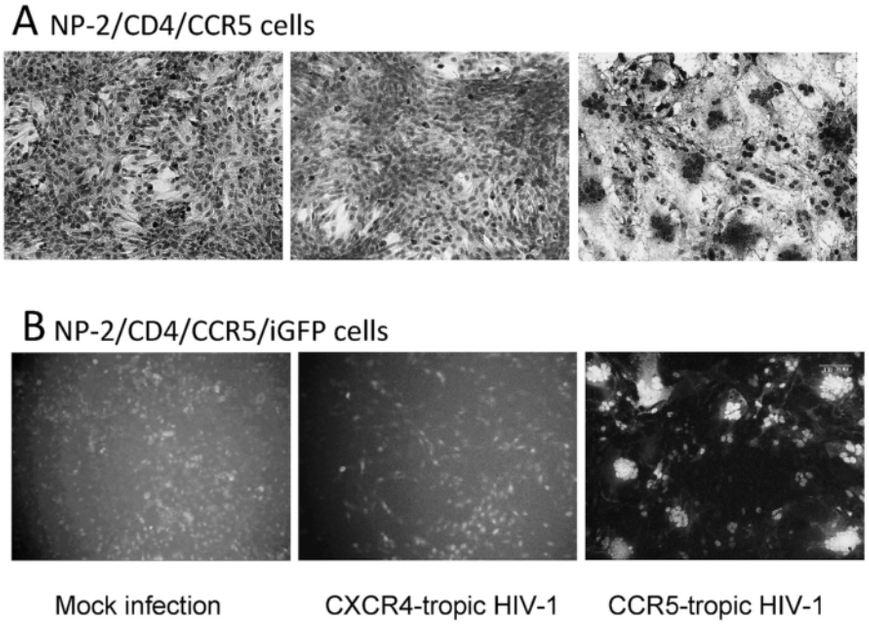

Fig. 6 Infection of NP-2/CD4/CCR5 cells with HIV-1.
(A) NP-2/CD4/CCR5 are cells highly susceptible to CCR5-tropic HIV-1 but completely resistant to CXCR4-tropic HIV-1.
(B) NP-2/CD4/ CCR5/iGFP cells were established after transfection of NP-2/CD4/CCR5 cells with the plasmid containing GFP gene fused with the nuclear localization signal of HIV-1 Rev. GFP is expressed under the control of HIV-1 long-terminal repeat (LTR) and transported into nuclei. Cell clones expressing GFP in the presence of Tat protein of HIV/SIV or after productive infection with HIV/SIV were selected and used for infection as described above.

human monocytic cells to establish a cell line persistently infected with HIV-1, as HTLV-1-positive T cells could not survive once HIV-1 infection spread among them. A scanning electron microgram of HIV-1-positive U937 cells was supplied from Dr. Tanami (Fig. 5).

Selection of the NP-2 cell line as an indicator cell line for HIV/SIV infection

We examined more than 20 human cell lines, including brain-derived cell lines after transduction with a CD4-expression vector, to determine whether they became susceptible to T-cell-tropic, dual-tropic or macrophage-tropic HIV-1 strains. Most cell lines became susceptible to T-cell-tropic (CXCR4-tropic) viruses, such as the IIIB strain. A few cell lines were still resistant to typical HIV-1 strains that we had tested, and the NP-2 cell line was among them.[26,27] No cell lines tested by us were susceptible to CCR5-

Table 1 Properties of the N-terminal regions of HIV/SIV coreceptors

GPCR	Amino acid sequence	Coreceptor activity	Tyrosine motifs		
			DY	NY	EY
1. Chemokine receptors					
CCR1	METPNTTEDYDETTEFDYGDATPCQKVNERAFGA	+	3	0	0
CCR2	MLSTSRSRFIRNTNESGEEVTTFFDYDYGAPCHKFDVKQIGA	+	3	0	0
CCR3	MTTSLDTVETFGTTSYYDDVGLLCEKADTRALMA	+	1	0	0
CCR4	MNPTDIADTTLDESIYSNYYLYESIPKPCTKEGIKAFGE	−	0	1	1
CCR5	MDYQVSSPIYDINYYTSEPCQKINVKQIAA	+	1	1	0
CCR6	MSGESMNFSDVFDSSEDYFVSVNTSYYSVDSEMLLCSLQEVRQFSRL	−	1	0	0
CCR7	MDLGKPMKSVLVVALLVIFQVCLCQDEVTDDYIGDNTTVDYTLFESLCSKK-DVRNFKAW	−	2	0	0
CCR8	MDYTLDLSVTTVTDYYYPDIFSSPCDAELIQTNGK	+	2	0	0
CCR9	MTPTDFTSPIPNMADDYGSESTSSMEDYVNFNFTDFYCEKNNVRQFASH	+	2	0	0
CCR10	MGTEATEQVSWGHYSGDEEDAYSAEPLPELCYKADVQAFSRAFQPSVSLTVA	+	0	0	0
CCR11	MALEQNQSTDYYYEENEMNGTYDYSQYELICIKEDVREFAKV	−	3	0	2
D6	MAATASPQPLATEDADSENSSFYYYDYLDEVAFMLCRKDAVVSFGKVFLP	+	2	0	0
CXCR1	MSNITDPQMWDFDDLNFTGMPPADEDYSPCMLETETLNK	+	1	0	0
CXCR2	MEDFNMESDSFEDFWKGEDLSNYSYSSTLPPFLLDAAPCEPESLEINK	+	0	1	0
CXCR3	MVLEVSDHQVLNDAEVAALLENFSSSYDYGENESDSCCTSPPCPQDFSLNFDR	+	2	0	0
CXCR4	MEGISIYTSDNYTEEMGSGDYDSMKEPCFREENANFNKI	+	2	1	0
CXCR5	MNYPLTLEMDLENLEDLFWELDRLDNYNDTSLVENHLCPATEGPLMASF-KAVFVP	+	0	3	0
CXCR6	MAEHDYHEDYGFSSFNDSSQEEHQDFLQFSKV	+	2	0	0
CXCR7	MDLHLFDYSEPGNFSDISWPCNSSDCIVVDTVMCPNMPNKSVLL	+	1	0	0
CX3CR1	MDQFPESVTENFEYDDLAEACYIGDIVVFGT	+	1	0	1
XCR1	MESSGNPESTTFFYYDLQSQPCENQAWVFAT	+	1	0	0
2. HIV/SIV coreceptors other than chemokine receptors					
APJ	MEEGGDFDNYYGADNQSECEYTDWKS	+	0	1	1
FPRL1	METNFSTPLNEYEEVSYESAGYTVLRI	+	0	0	3
GPR1	MEDLEETLFEEFENYSYDLDYYSLESDLEEKVQLGVVHWVSI	+	2	1	0
GPR15	MDPEETSVYLDYYYATSPNSDIRETHSHVPYTS	+	1	0	0

The N-terminal amino acid sequences of the chemokine receptors and non-chemokine receptors having HIV/SIV coreceptor activities, HIV-1 coreceptor activities and the presence of DY (DY or YD), NY (NY or YN) and EY (EY or YE) motifs are shown.

tropic HIV-1.

When NP-2/CD4 cells were further transduced with CCR5 or CXCR4, they became highly susceptible to CCR5-tropic or CXCR4-tropic HIV-1 strains, and large syncytia often formed a few days after inoculation of HIV-1, HIV-2 or SIV[27] (Figs. 1D, 6 and 10B). To detect new HIV/SIV coreceptors, we transduced many GPCR genes and found that chemokine receptors, such as CCR8, CXCR5, and XCR1, or the non-chemokine receptors FPRL1, GPR1, and RDC1 were new types of coreceptors for HIV-1, HIV-2 or SIV.[28-34] Fig. 7 shows the phylogenetic relationship between the HIV/SIV coreceptors and chemokine receptors. Most chemokine receptors can function as the coreceptors (Table 1).

Establishment of NP-2 cells with inducible GFP (iGFP) cells

Several indicator cell systems in which indicator proteins, such as βgalactosidase or green fluorescent protein (GFP), are induced after the establishment of infection of HIV or SIV have been reported. However, it has been difficult to accurately determine CCR5 or CXCR4 usage in HIV/SIV strains using the indicator cell systems like the HeLa or HOS cell line reported previously because these cell lines are already expressing HIV/SIV co-receptors other than CCR5. Systems using T-cell lines, such as H9 and CEM can detect CXCR4-tropic HIV-1 but not CCR5-tropic HIV-1.

To work around these limitations, we have established an indicator cell system using the NP-2 cell line and GFP for direct detection of cells infected with HIV and SIV and for strict determination of their CCR5 and CXCR4 use. After transfection with a plasmid containing HIV-1 long terminal repeat and the green fluorescent protein (GFP) gene, the NP-2 indicator cells with inducible GFP (NP-2/iGFP cells) were established from cell clones that were mostly negative for GFP but that had become positive for GFP after HIV-1 infection, namely, after production of the HIV/SIV transactivator protein Tat.[35] One day after the infection of these cells with HIV-2 or SIV and HIV-1, GFP-positive cells could be identified (Fig. 6B). The indicator cells formed GFP-positive syncytia 12 hours after cocultivation with HeLa cells expressing HIV-1

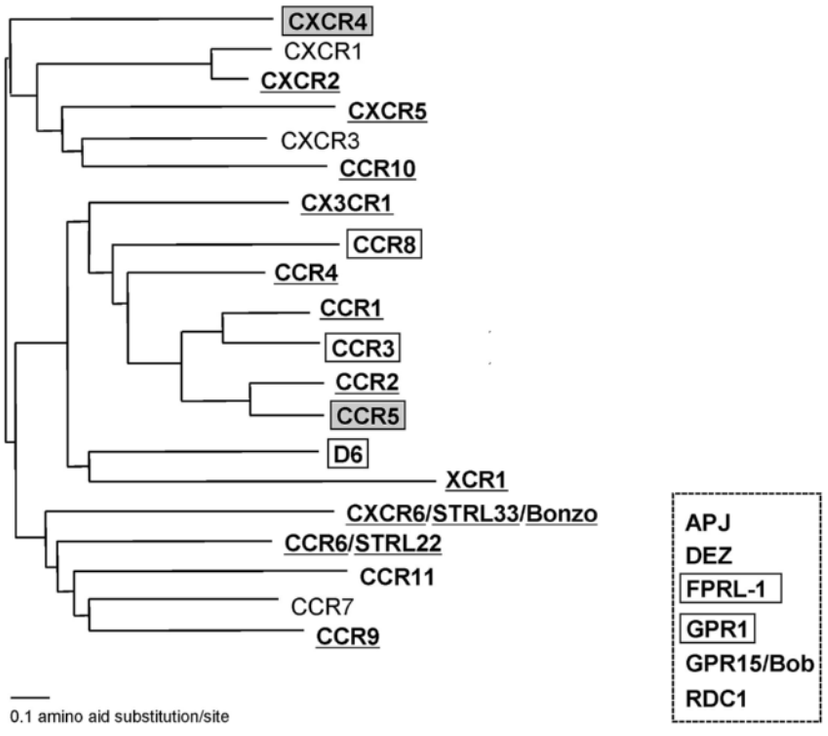

Fig. 7 Phylogeny of the chemokine receptor genes and the HIV/SIV coreceptors not belonging to them. The chemokine receptors reported to function as HIV/SIV coreceptors are boxed or underlined. The major coreceptors CCR5 and CXCR4 and other so-called minor coreceptors relatively used by primary HIV-1 strains according to our analyses are boxed.

Env and Tat proteins.[36] We have introduced this type of coculture experiments into the curriculum for medical students: it can be safely done by them and will help them to understand the infection mechanism of HIV-1. The NP-2/iGFP indicator cells enabled us to detect a small number of cells or a single focus of cells infected with HIV and SIV, to monitor the spread of their infection and to determine their CCR5/CXCR4 usage without cell fixation.

Lack of the trans-receptor mechanism in HIV-1 infection

We established NP-2 cells expressing CD4, CCR5 or CXCR4 alone to examine whether NP-2 cells expressing CD4 or a coreceptor alone were more susceptible to cell-free virus infection or syncytium formation induced by HIV-1 Env than NP-2 cells negative for both. For this purpose, we used cocultures of NP-2 cells expressing CD4 and those expressing CCR5 or CXCR4 and infected them with HIV-1 or cocultured these NP-2 cells with HIV-1 Env-expressing cells.[35]

It has been previously reported that HIV-1 can infect cells using CD4 expressed on the surface of one cell and CCR5 or CXCR4 expressed on another cell. This type of infection has been referred to as the trans-receptor mechanism of virus infection[37] (Fig. 1E). According to the original report, up to 4% of CD-4-negative, coreceptor-positive ($CD4^{(-)}$ $coreceptor^{(+)}$) adherent cells can be infected with HIV-1 when CD4-positive human T cells had been cocultured with these adherent cells and infected with HIV-1.

We tried to confirm this mechanism using similar types of cells[36] as those used in that report. That is, we used several assay systems to examine whether HIV-1 can infect $CD4^{(-)}$ $coreceptor^{(+)}$ cells via CD4 expressed on neighboring cells. Firstly, C8166 human T cells, expressing CD4 but not CCR5, were mixed with NP-2/CCR5/iGFP ($CD4^{(-)}$ $coreceptor^{(+)}$ cells) or NP-2/iGFP ($CD4^{(-)}$ $coreceptor^{(-)}$ cells as control) indicator cells, and inoculated with a CCR5-tropic HIV-1 strain. No GFP-positive cells ($<0.01\%$) were detected in these iGFP cells after 3 days post-inoculation (Fig. 8A). In contrast, NP-2/CD4/CCR5/iGFP cells ($CD4^{(+)}$ $coreceptor^{(+)}$ control cells) alone or mixed with C8166 cells yielded numerous GFP-positive cells and syncytia after infection (Fig. 8A). Thus, our data indicate that HIV-1 cannot infect $CD4^{(-)}$ $CCR5^{(+)}$ cells using the CD4 of adjacent cells.

We also confirmed this using DNA PCR. NP-2/CCR5/iGFP, NP-2/iGFP, or NP-2/CD4/CCR5/iGFP cells were mixed with C8166 cells, and inoculated with a CCR5-tropic HIV-1 strain. After being in culture for 2 days, cellular DNAs were isolated and subjected to PCR. No HIV-1-specific PCR products

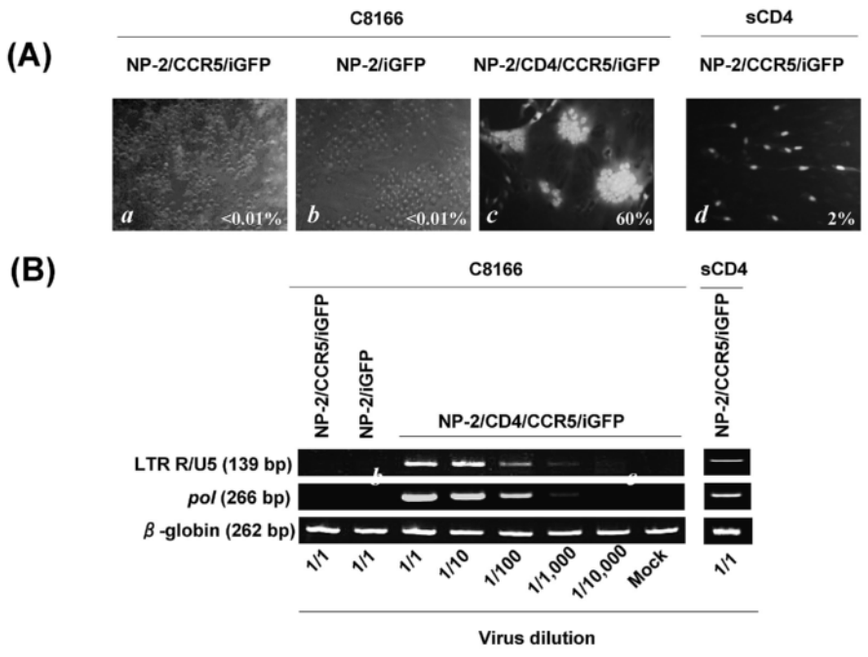

Fig. 8 Lack of trans-receptor mechanism of HIV-1 infection.
(A) CD4-positive C8166 cells overlaid onto NP-2/CCR5/iGFP, NP-2/iGFP and NP-2/CD4/CCR5 cells and infected with CCR5-tropic HIV-1 strain. NP-2/CCR5 cells were infected with HIV-1 in the presence of sCD4 (10μg/ml). The cells were examine three days after HIV-1 inoculation.
(B) Mixtures of C8166 cells with NP-2/CCR5/iGFP, NP-2/iGFP and NP-2/CD4/CCR5 cells were infected with undiluted (1/1) or serially diluted HIV-1 as described above. HIV-1 DNA was detected 2 days after inoculation by DNA PCR specific for HIV-1.

were detected using NP-2/CCR5/iGFP and NP-2/iGFP cell samples (Fig. 8B). HIV-1-specific PCR systems were sensitive enough to detect PCR bands when a mixture of NP-2/CD4/CCR5/iGFP and C8166 cells had been infected with 1/1,000-diluted virus. Thus, these results do not support that the trans-receptor mechanism of HIV-1 takes place frequently. We conclude that the expression of CD4 and a suitable coreceptor on the same cell is essential for the establishment of HIV-1 infection under our culture conditions.

We also reported that some HIV-2 strains can efficiently infect $CD4^{(-)}$ $coreceptor^{(+)}$ NP-2 cells in the presence of soluble CD4 (sCD4).[38] Thus, NP-2/CCR5/iGFP cells were infected with CCR5-tropic strain in the presence of sCD4. Approximately 2% of NP-2/CCR5/iGFP cells expressed GFP on day 3 post-infection (Fig. 8A). These cells also gave discrete HIV-1-specific PCR bands (Fig. 8B). Collectively, our data demonstrate that sCD4, but not cell-associated CD4, can help HIV-1 to infect $CD4^{(-)}$ $coreceptor^{(+)}$ cells.

Incorporation of HIV-1 resistant cells into syncytia induced by HIV-1

HIV-1-resistant cells expressing either CD4 or a coreceptor are often found surrounding HIV-1-susceptible cells, expressing both CD4 and a compatible coreceptor, in vivo. When HIV-1-resistant NP-2 cells expressing CD4 or a coreceptor or lacking both were mixed with $CD4^{(+)}$ $coreceptor^{(+)}$ NP-2 cells and inoculated with HIV-1, a small number of all these HIV-1-resistant cells (up to 2% under our assay conditions) were similarly incorporated into the syncytia induced by HIV-1, indicating a CD4- and coreceptor-independent incorporation of HIV-1-resistant cells into the syncytia. This incorporation was significantly impaired by the transfection of these cells with siRNAs for adhesion molecules, integrin β1 or cadherin-11. Our study demonstrates that HIV-1-resistant cells can be incorporated into syncytia induced by HIV-1 and this incorporation may partially be mediated through the adhesion molecules, although infection of HIV-1 resistant cells with cell-free HIV-1 will hardly take place even when these cells are co-cultured with HIV-1-susceptible cells.[36] Syncytia have often been detected in the brain of HIV-1-infected subjects. The presence of syncytia is reported to be a hallmark of HIV-1 replication in the brain.[39,40]

Promiscuous relationship between chemokines and their receptors

As shown in Fig. 9, many chemokines will bind to multiple chemokine receptors. MIP-1, MCP-2,

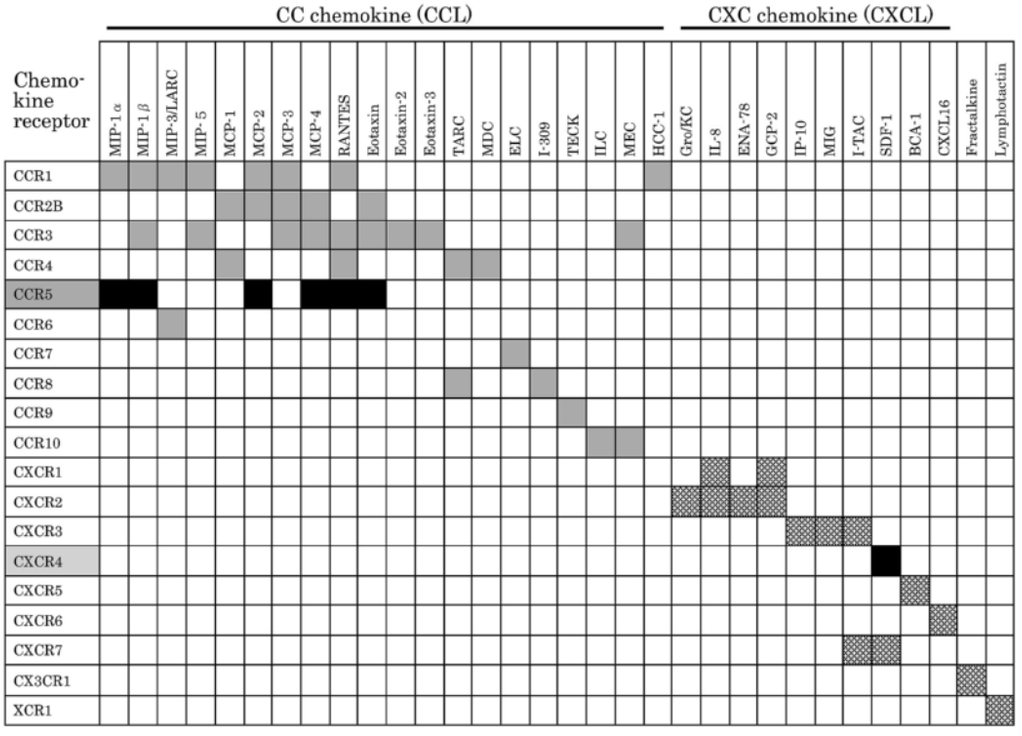

Fig. 9 The chemokine receptors and their major ligands. CC chemokine (CCL) receptors, CXC chemokine (CXCL) receptors and CX3C and XC chemokine receptors are shown. Several CCRs recognize multiple ligands.

MCP-4, RANTES, etc. will bind to CCR5. This relationship has been called 'promiscuous'. I propose that this property is important for permitting the HIV-1 variability and its persistence in humans. HIV-1 is highly mutable, especially in its V3 region; however, this region should also recognize a co-receptor, such as CCR5 (Figs. 1 and 2).

Generally speaking, a region necessary for an important function should not be mutable. Because CCR5 is promiscuous in its ligands, it may recognize HIV-1 strains with mutated V3 sequences. The V3 region is also known to be a major immunodominant domain, and human antibodies or mouse monoclonal antibodies that can neutralize HIV-1 can recognize this region. Thus, it is expected that HIV-1 mutants that can still recognize CCR5 can readily become immune escape mutants.[41] The use of chemokine receptors, such as HIV/SIV coreceptors, may favor the survival and propagation of HIV/SIV mutants.

Use of the co-receptors by HIV-1 present in humans

We have made a panel of NP-2/CD4 cells expressing various coreptors, to determine which coreceptors are used by HIV-1 present in humans.[42] We have established NP-2/CD4 cells expressing CCR1, CCR2b, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9b, CCR10, CCR11, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, XCR1, D6, APJ, FPRL1, GPR1, RDC1, etc.

Then, this panel of NP-2 cells was cocultivated with PBMC samples of 17 HIV-1-infected Japanese patients and examined for the establishment of HIV-1 infection by immunofluorescence assays to HIV-1 antigens. Twelve PBMC samples gave positive results. HIV-1 antigen-positive NP-2/CD4/CCR5 cells were obtained when cocultured with nine PBMC samples. Five and four samples gave positive results with the NP-2/CD4/CXCR4 and NP-2/CD4/GPR1 cells, respectively.[34]

The HIV-1 virus present in the culture supernatants of the NP-2/CD4/CCR5 or NP-2/CD4/CXCR4 cells that were positive for HIV-1 antigens were grown on NP-2/CD4 cells expressing CCR1, CCR3, CCR8, D6 or FPRL1. Thus, in addition to the chemokine receptors CCR5 and CXCR4, the chemokine receptors CCR8 and D6 and the non-chemokine receptors GPR1 and FPRL1 were used by HIV-1 infections in humans. NP-2 cells will be useful for isolating the primary HIV-1 strains from patients.[34]

Characterization of HIV-1 variants that can grow in U87/CD4 cells

I have been interested in HIV-1 replication in the human brain because neurological disorders are fre-

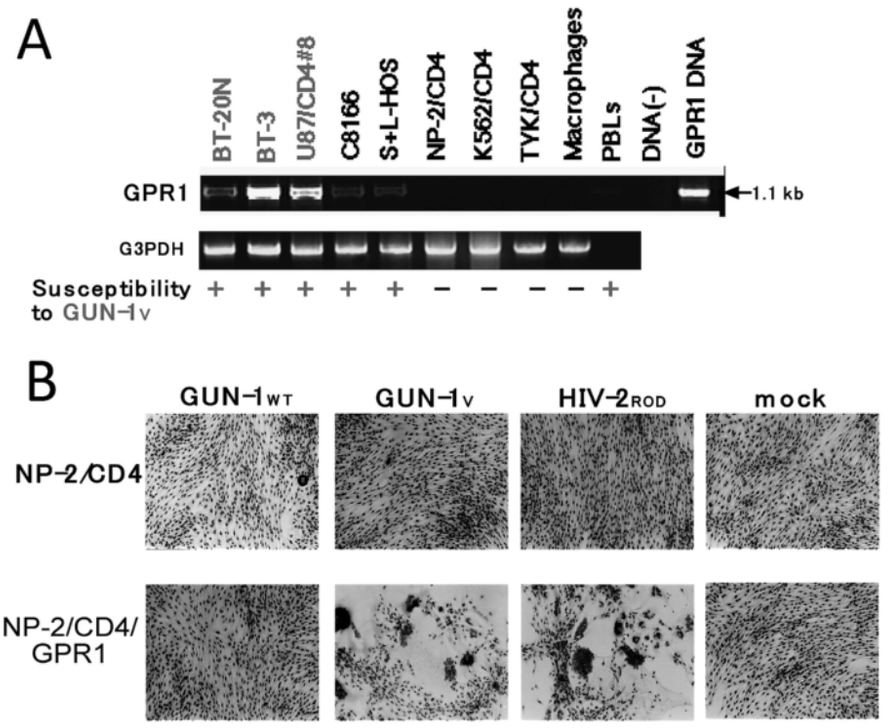

Fig. 10 GPR1 as a new type of HIV-1 coreceptor.
(A) Detection of GPR1 RNA by RT-PCR. The brain-derive fibroblast-like cells, BT20-N and BT3, glioblastoma cells transduced with CD4, U87/CD4, HTLV-1-positive and CD4-positeve T-cells, C8166, and human osteosarcoma cells, S+L-HOS, are positive for GPR1 RNA. These cells are susceptible to a variant of GUN-1wt strain, GUN-1v.
(B) NP-2/CD4/GPR1 cells are susceptible to an HIV-1 strain, GUN-1v, and an HIV-2 strain, ROD, and formed many syncytia in a few days after infection. NP-2/CD4 cells are resistant to all HIV/SIV strains including GUN-1 and ROD we have tested.

quently found in humans or animals infected with retroviruses.[39,40] HTLV-1 causes HTLV-1-associated myelopathy (HAM) and adult T-cell leukemia (ATL). Nearly half of the HIV-1-infected subjects with or without immunodeficiency have been reported to develop some type of neurological disorder during the clinical course of infection with HIV-1.

Thus, we established several glioma cell lines transduced with CD4, including NP-2/CD4 and U87/CD4, and examined whether these cells are infectable with HIV-1 as described above. The majority of glioma cell lines expressing CD4 were susceptible to CXCR4-tropic (X4) HIV-1 strains, although U87/CD4 and NP-2/CD4 cells were highly resistant to HIV-1 strains. We had repeatedly cocultivated U87/CD4 cells with human T cells infected with HIV-1 GUN primary isolates and in some cases, variant viruses were isolated.[43,44] No viral mutants that could grow in NP-2/CD4 cells, however, have been isolated by us so far.

We isolated HIV-1 variants, such as GUN-1v, GUN-4v and GUN-7v, from the GUN-1wt, GUN-4wt and GUN-7wt wild-type viruses, respectively, and then determined the variation of the genomic sequence

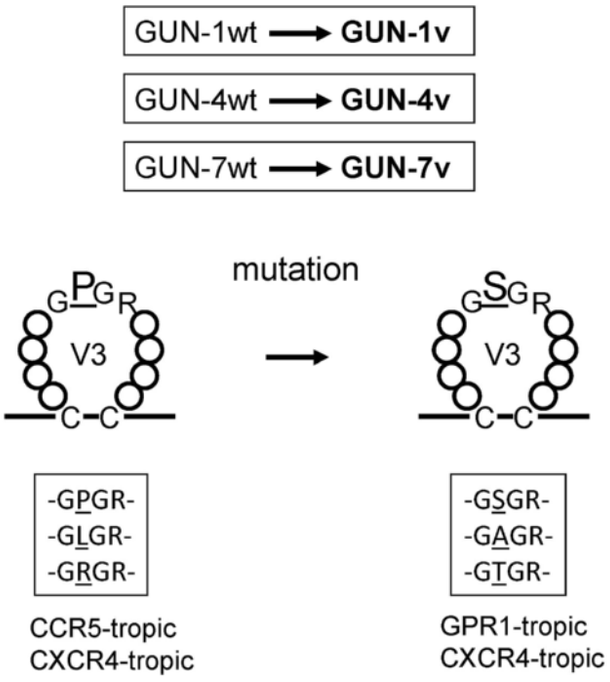

Fig. 11 Isolation of GUN-1, GUN-4 and GUN-7 variants that can grow in U87/CD4 cells and production HIV-1 point-mutants at the tip of V3 region. The single point mutations affect coreceptor use from CCR5 to GPR1.

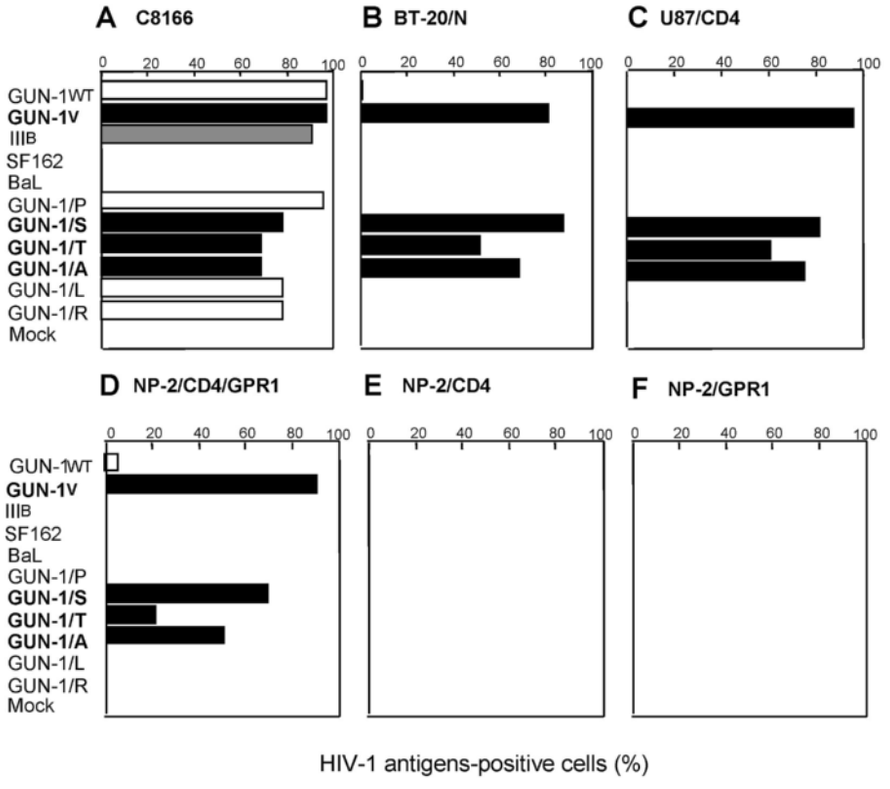

Fig. 12 We isolated HIV-1 variants, GUN-1v, which could grow in U87/CD4 cells, from GUN-1wt strains. IIIB is CXCR4-tropic HIV-1 and SF162 and BaL are CXCR5-tropic HIV-1. GUN-1/P, GUN-1/S, GUN-1/T, GUN-1/A, GUN-1/L and GUN-1/R are point mutants at the tip of V3 region as shown in Fig. 12. C8166, BT-20N, U87/CD4, NP-2/CD4/GPR1, NP-2/CD4 and NP-2 GPR1 cells were infected with HIV-1 strains and examined by indirect immunofluorescence using HIV-1-positive human sera as the first antibody. GUN-1/S, GUN-1/T, and GUN-1/A, plated onto BT-20N, U87/CD4 and NP-2/CD4/GPR1 cells as well as the human T-cell line C8166 expressing CXCR4 but not CCR5. IIIB, BaL and SF162 strains did not plated onto BT-20N, U87/CD4 and NP-2/CD4/GPR1 cells.

of the GUN viruses (Fig. 11). We identified the genetic variation responsible for this phenotype, which turned out to be a single point mutation at the tip of the V3 region, from GPGR to GSGR (from proline to serine) of GUN-1wt and GUN-1v. Sequence analyses revealed that in the cases of the GUN-4v and GUN-7v strains, GPGR to GAGR and GPGR to GTGR point mutations, respectively, were present.

Identification of GPR1 as a coreceptor in infection of brain-derived cells with HIV-1 variants

We established NP-2/CD4 cells expressing GPR1 and compared their susceptibility to GUN-1v variants and GUN-1 mutants, GUN-1/S, GUN-1/T, GUN-1/A, GUN-1/L and GUN-1/R, harboring point mutations at the tip of the V3 region (Fig. 11). NP-2/CD4/GPR1 and U87/CD4 cells and brain-derived fibroblast-like cells (BT-20N cells) showed a similar HIV-1 susceptibility pattern to the HIV-1 variants and mutants (Fig. 12). These results indicate that a single point mutation in the V3 region can determine the coreceptor usage or cell tropism of HIV-1.

We also examined GPR1 expression in various cells and their susceptibilities to GUN-1 variants and mutants. Figure 10A shows that cells expressing GPR1, BT-20N, BT-3, U87/CD4, C8166 and S+L-HOS, are susceptible to GUN-1v virus. These findings indicate that GPR1 is a coreceptor in the infection of brain-derived cells.[43-45] NP-2/CD4/GPR1 cells were also susceptible to the ROD strain of HIV-2 (Fig. 10B). Many HIV-2 and SIV strains use GPR1 as a coreceptor.

Inhibition of HIV-1 infection by the N-terminal peptide of GPR1

As we noticed that the N-terminal sequences of GPCRs were important for the function of the HIV/SIV coreceptors (Table 1), these sequences were expected to bind to HIV/SIV virions. Therefore, we synthesized the N-terminal peptides of the coreceptors CCR5, CXCR4 and GPR1 and examined whether these peptides could affect HIV-1 infection.[46] Unexpectedly, only the GPR1 peptide, consisting of N-terminal 27 amino acids, but not the others, inhibited infection of cells with not only GPR1-tropic viruses,

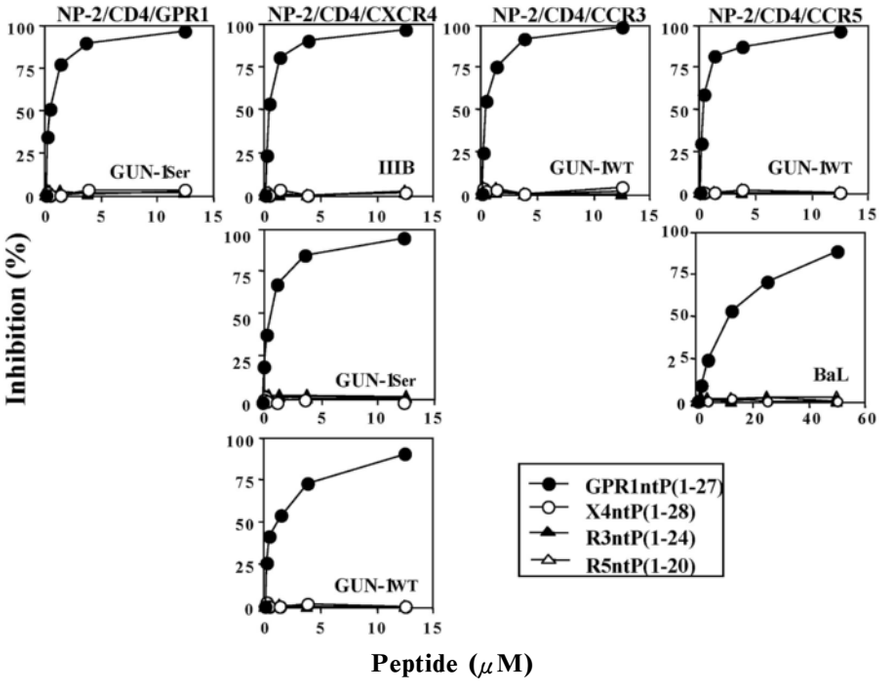

Fig. 13 Effects of N-terminal peptides of the coreceptors on HIV-1 infection. The 27 amino-acid peptide of GPR1 N-terminal, but not the N-terminal sequence of CCR5 (R5), CXCR4 (X4) or CCR3 (R3) peptide, markedly inhibited infection of NP-2/CD4/GPR1, NP-2/CD4/CXCR4, NP-2/CD4/CCR3 and NP-2/CD4/CCR5 cells with various HIV-1 strains, such as IIIB, GUN-1wt, GUN-1Ser or BaL.

but also R5 and X4 viruses and R5-X4 dual-tropic viruses (Fig. 13).

Trial to identify new HIV/SIV co-receptors

Approximately 20 GPCRs have been shown to function as HIV/SIV coreceptors[34,42] (Fig. 7). There may be another coreceptor belonging to GPCR family because it is one of the largest gene families present in the human genome. We have examined the N-terminal regions of the reported HIV/SIV coreceptors and noticed the frequent presence of tyrosine (Y) associated with aspartic acid (D), asparagine (N) or glutamic acid (E). Namely, tyrosine motifs, DY, NY, or EY, are often found in the NTRs of the coreceptors (Table 1).

After screening more than 900 different GPCRs, we selected 13 eligible candidates, including CCR6, CKR-L3, DRD, G2A, GPR12, GPR25, HCRTR2, LPAR2, LPAR3, NPY5R, OXGR1, PROKR1 and PROKR2. DNA microarray analyses of NP-2/CD4/CCR5 cellular RNA showed that these cells abundantly express GPR12, NPY5R, OXGR1 and PROKR1. Because NP-2/CD4 cells were highly resistant to all of the HIV/SIV strains we tested, these GPCRs would not function as an HIV/SIV co-receptor under our assay conditions. The other candidate GPCRs, CCR6, CKR-L3, DRD2, G2A, GPR25, HCRTR2, LPAR2, LPAR3 and PROKR2 are now under investigation. Although so-called minor coreceptors may not play an important role in HIV-1 infection, this type of information will give us insight into why HIV/SIV uses GPCRs as coreceptors for infection.

We have sent NP-2 cell sublines expressing one of the HIV/SIV coreceptors as well as CD4, especially NP-2/CD4/CCR5 and NP-2/CD4/CXCR4, to many researcher groups in Japan and several countries as shown by several references listed below.[22,47-51] We are pleased to send them if requested.

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