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Evolution of coreceptor use and CD4-independence in envelope clones derived from SIVsm-infected macaques

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Abstract

Coreceptor use of HIV can evolve during infection. We previously examined coreceptor use of related SIVsm inoculum viruses and sequential reisolates from cynomolgus macaques. These viruses exhibited broad coreceptor specificities and, generally, CCR5 use remained efficient and stable, while alternative coreceptor use decreased longitudinally. Here we demonstrate that individual envelopes (Envs) from inoculum and reisolate viruses fuse via a range of coreceptors, including CCR5, CCR8, CXCR6, GPR15, GPR1, and APJ. On the whole, coreceptor use of Envs from sequential reisolates recapitulated that of reisolate viruses, thus CCR5 use remained stable while alternative coreceptor use tended to decrease over time. Rhesus CCR5, GPR15, and CXCR6 supported fusion to a similar extent as their human counterparts. Additionally, a number of Envs mediated CD4-independent fusion via CCR5 and GPR15. Envs from different inoculum viruses exhibited distinct dependencies on CD4 for fusion via CCR5, ranging from strictly CD4-dependent to efficiently CD4-independent. Early reisolates from macaques infected with CD4-independent inoculums maintained or evolved Envs with a broad range of CD4-independence. CD4-independence became less variable/efficient in late reisolates from macaques that developed neutralizing antibodies. Infection with a CD4-dependent virus resulted in evolution of CD4-independent Envs in late reisolates. While CD4 independence can potentially broaden tropism in vivo, CD4-independent viruses are particularly sensitive to neutralizing antibodies. Therefore, interplay between receptor tropism and neutralization may shape viral evolution and SIV pathogenesis.

Introduction

Simian immunodeficiency virus (SIV) infection of macaques is often used as a model for human immunodeficiency virus (HIV) infection of humans. In experimental SIV infection the time and route of infection as well as the inoculum virus and the infectious dose are controlled. Another advantage of the macaque system is that events occurring immediately after infection can be followed. In humans, early sequelea of HIV infection can rarely be studied since the onset of symptoms, if any at all, can occur several weeks after infection.

HIV or SIV infection begins with an acute phase characterized by a high level of virus replication resulting in a peak viral load in the infected individual. Subsequently, there is a characteristic decline in viral load to a steady-state level, called the viral set point, which is predictive of the severity of infection and disease course (Ho, 1997; Musey et al., 1997; Rinaldo et al., 1995). Decline in viral load is a consequence of activation of the host's immune response, which controls viral replication to varying degrees during the asymptomatic phase of infection (Koup et al., 1994; Schmitz et al., 1999). With increasing destruction of the

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immune system, the balance between host control and virus replication is lost leading to increased plasma viral load, accelerated loss of $CD4^+$ cells, and clinical symptoms of immunodeficiency.

To enter target cells, HIV and SIV usually require an interaction with both CD4 and a secondary coreceptor molecule on the cell surface (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). Viruses isolated during the asymptomatic phase of HIV-1 infection most often use the chemokine receptor molecule CCR5 (R5 viruses; Berger et al., 1998). During progressive disease approximately 50% of HIV-1-infected individuals acquire viruses that utilize a related chemokine receptor, CXCR4, either in place of (X4 viruses) or in addition to CCR5 (R5X4 viruses) (Björndal et al., 1997; Scarlatti et al., 1997). SIV isolates from infected monkeys (SIVsm and SIVmac of sooty mangabey origin, isolated from sooty mangabey or macaque, respectively) also use CCR5 as coreceptor (Chen et al., 1997; Edinger et al., 1997a; Marcon et al., 1997). In addition, they often use CXCR6 (formerly STRL33/Bonzo) and/or GPR15/BOB (Deng et al., 1997; Edinger et al., 1998; Farzan et al., 1997). However, unlike HIV-1, progressive SIV disease is not associated with a change in viral tropism to CXCR4 use. In general, CXCR4 use is rare among SIV strains, and at least in one case was the result of extensive in vitro passage in human peripheral blood lymphocytes (Owen et al., 2000; Schols and De Clercq, 1998; Vödrös et al., 2001). In addition, reisolated viruses from macaques infected with a CXCR4 using isolate lost the ability to use CXCR4 (Vödrös et al., 2001), suggesting control of CXCR4-using viruses in the monkey host or selection for reduced coreceptor specificity.

While primary HIV-1 isolates are almost exclusively CD4-dependent, CD4-independent use of coreceptors by HIV-2 (Endres et al., 1996; Liu et al., 2000; Reeves et al., 1997, 1999) and SIV strains (Edinger et al., 1997b, 1999; Liu et al., 2000; Reeves et al., 1997; Schenten et al., 1999) has been reported. In addition, several HIV-1 strains have been selected for CD4-independent use of either CCR5 or CXCR4 (Dumonceaux et al., 1998; Hoffman et al., 1999; Kolchinsky et al., 1999; LaBranche et al., 1999). Since T-cell line-adapted CD4-independent HIV-1 strains and SIV variant envelopes are highly sensitive to inhibition by neutralizing antibodies (Edwards et al., 2001; Kolchinsky et al., 2001; Puffer et al., 2002), the humoral immune response may control the emergence of CD4-independent variants in vivo.

We previously reported that a subset of coreceptor molecules could mediate infection of variant SIVsm strains and sequential reisolates obtained from infected cynomologus macaques (Edinger et al., 1997b, 1999; Liu et al., 2000; Reeves et al., 1997; Schenten et al., 1999). We observed stable use of CCR5 by inoculum and reisolated viruses and declining utilization of CXCR6, GPR15, and CXCR4 over time. To extend these studies, we cloned envelope genes from inoculum viruses and reisolates from SIVsm-infected cynomolgus macaques that exhibited fast or slow disease progression and in which the neutralizing antibody response was known. We examined Env clones for their ability to mediate fusion on an extended panel of coreceptor molecules, enabling us to determine if broad coreceptor tropism was a feature of individual Env clones or resulted from a virus swarm of mixed coreceptor specificities. CD4-independent use of coreceptors was also addressed by analyzing the fusion capacity of envelope clones in the presence or absence of CD4.

Results

Cloning functional SIV envelopes from inoculum viruses and sequential reisolates

We have previously studied the coreceptor use of related SIVsm inoculum viruses (SIVsmA, SIVsmB, and SIVsmC) with different passage histories (detailed in the materials and methods section and summarized below) and sequential viral reisolates from cynomolgus macaques infected with these viruses (Vödrös et al., 2001). Inoculum viruses originated from the SIVsm isolate SMM-3, obtained from a naturally infected Sooty mangabey (Vödrös et al., 2001). SIVsmA originated from SMM-3 that was passaged twice on HUT-78 cells, followed by two passages on human (h) PBMCs. SIVsmB was derived by further passage of SIVsmA on hPBMCs for 3 months. SIVsmC was isolated on hPBMCs by coculture with PBMCs from a symptomatic cynomolgus macaque that had been inoculated with SIVsmA. Inoculum and reisolated viruses exhibited broad coreceptor specificities (Vödrös et al., 2001), detailed in the material and methods section and Table 1. In general, CCR5 use of inoculum and reisolated viruses remained efficient and stable, while the efficiency of alternative coreceptor use decreased over time.

Env genes were cloned from inoculum and reisolate viruses to determine whether the coreceptor tropism of these viruses was conferred by individual Env genes or Env genes of different coreceptor specificities. Viral envelope genes, amplified in two to four independent PCR reactions from genomic DNA isolated from virus-infected human PBMC (described in detail in the materials and methods section) were cloned into the pCI-PRE expression vector. On average, four or five clones from each independent PCR reaction were analyzed; 88% of clones (168 of 191) screened by restriction digestion contained Env gene inserts of expected size. Clones containing appropriate sized inserts were tested for function in a cell-cell fusion assay. Since all viral isolates were previously shown to use CCR5 efficiently and stably as a coreceptor for infection of GHOST(3) cells (Vödrös et al., 2001), the ability of Env clones to fuse with CD4/$CCR5^+$ cells was determined (Fig. 1, and data not

Table 1
Characteristics of SIVsm inoculated monkeys: coreceptor use of sequential reisolates and neutralizing antibody production

Monkey	SIVsm inoculum[a]	Death with immonudeficiency (days PI)	Virus isolation (days)[b]	Corecepor use of reisolates[c]			Serum neutralizing activity[d]		
				Early	Middle	Late	Against isolate	At indicated day	Reciprocal titer
MF8	A	541	**12**-137-**541**	CCR5, CXCR6, GPR15	CCR5, CXCR6, GPR15	CCR5 [GPR15]	inoculum	d137	160
							12d	d137	>640
MF10	A	541	**12**-226-541	CCR5, CXCR6, GPR15	CCR5, CXCR6, GPR15	CCR5, CXCR6, GPR15	inoculum	d226	40
							12d	d226	320
H9	B	174	**12-174**	CCR5, CCR3, CXCR4, CXCR6, GPR15		CCR5, CXCR6, GPR15	inoculum	d174	320
							12d	d174	>320
H56	B	898	12-217-**365**-538-**898**			CCR5, GPR15	inoculum	d217, d365, d538	>320
							12d	d217	320
							12d	d365	40
							12d	d538	80
							217d	d365, d538	20
							365d	d538	40
							538d	d538	80
H27	C	145	**12-145**	CCR5, GPR15 [CXCR6]		CCR5, GPR15		none	
H28	C	607	**12**-125-365-**607**	CCR5, GPR15 [CXCR6]	CCR5, GPR15 [CXCR6]	CCR5 [GPR15]	inoculum	d125	80
							12d	d125, d365	40
							125d	d365	>320

[a] Inoculum A and C were used undiluted, H9 received $>10^5$ monkey infectious dose-50 (MID_{50}), while H56 was inoculated with 10^1–10^2 MID_{50} of inoculum B.

[b] Isolates underlined were tested for coreceptor use; envelopes were cloned from isolates in bold face.

[c] Summarized from Vödrös et al. (2001); CCR3, CCR5, CXCR4, CXCR6 and GPR15/BOB coreceptor utilization determined on CD4+ GHOST(3) cell lines. Weak use of coreceptor is indicated by parenthesis. Early, middle, late refers to reisolation date tested (see [b]).

[d] Summarized from data based on Zhang et al. (1993).

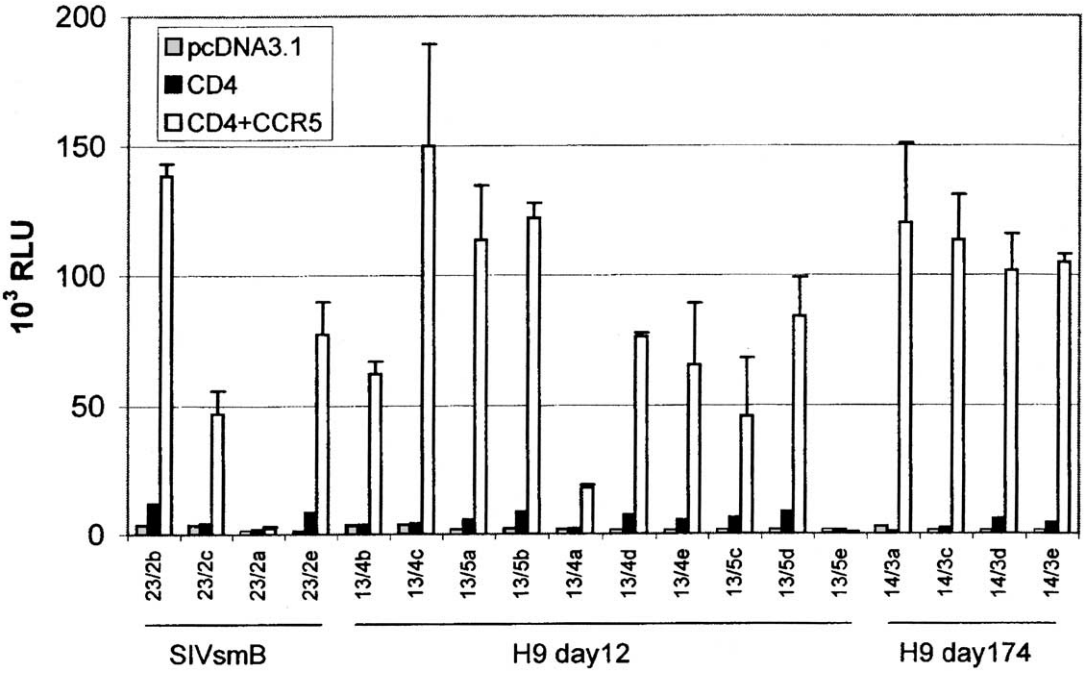

Fig. 1. Functional screening of viral Env clones. Fusion between Env-expressing effector cells (infected with a vaccinia virus encoding T7 polymerase) and receptor-expressing target cells (cotransfected with a T7-luciferase reporter plasmid). Fusion of effector and target cells results in luciferase expression. RLU, relative light units indicating luciferase activity; pcDNA3.1, control plasmid. Results represent fusion of Env clones from inoculum and reisolate viruses from one animal (H9).

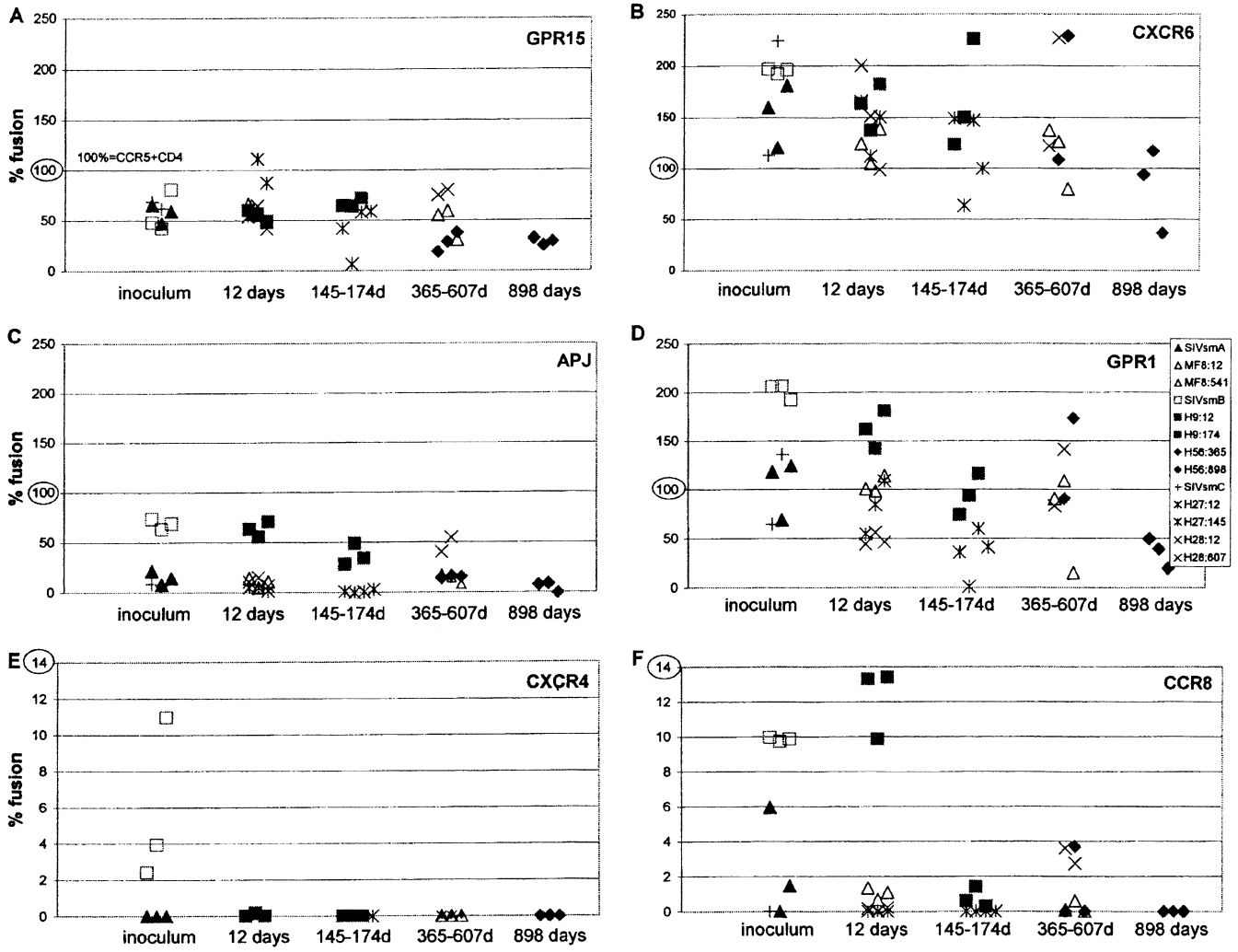

Fig. 2. Coreceptor use of envelope clones obtained from inoculum, early and late viral reisolates from SIVsm-infected cynomolgus macaques. The coreceptor use of two to four Env clones from each animal at each time point were analyzed. % Fusion, fusion between effector cells expressing indicated Envs and target cells expressing CD4 and indicated coreceptor, expressed as a percentage of fusion via target cells expressing CD4 and CCR5. Time points of virus reisolations are indicated. Coreceptor use of clones from macaque MF8 and H9 represent an average of three assays, coreceptor use of clones from macaque H27 represent an average of two assays, and the coreceptor use of clones from macaque H28 and H56 represent the average of replicates from individual experiments. The *y*-axes of panels A–D are plotted at equivalent scales (100% fusion value circled). The *y*-axes of panels E and F are plotted to a maximum value of 14% fusion (circled).

shown); 86% of Env clones tested (101 of 118) were fusion competent. Selected functional clones were then subjected to further detailed analysis, as described below.

Coreceptor use of SIVsm envelope clones from inoculum and reisolated viruses

The coreceptor use of selected envelopes cloned from SIVsm inoculum viruses as well as from early (12 days postinfection) and late (time of overt immunodeficiency) reisolates from five cynomolgus macaques was examined. QT6 quail cells transfected with envelope clones were assayed for their ability to fuse with cells expressing CD4 and various coreceptors. Since all SIV isolates were shown to use CCR5 efficiently in the presence of CD4, the fusion capacity of Env clones via other coreceptors was expressed as a percentage of fusion via CCR5 (Fig. 2). All Env clones were multicoreceptor tropic with efficient fusion detected via a number of coreceptors, with one exception: clone 16/1d (an individual clone from a day 145 reisolate of monkey H27) used only CXCR6 in addition to CCR5, while almost all other Envs mediated fusion via CXCR6, GPR15, and frequently GPR1 in addition to CCR5. In general, the coreceptor use of clonally derived envelopes was thus similar to the pattern of infection observed with the corresponding isolates on GHOST(3) cells (Table 1), in that both clones and biological isolates could use CCR5, CXCR6, and GPR15. Using the cell-cell fusion assay, we observed that Env clones derived from both SIVsmB-infected an-

imals and one SIVsmC-infected monkey showed a decline in use of certain alternative receptors compared to the inoculum virus. This is consistent with our earlier study in which we noted a decline in use of these receptors (especially CXCR6) when virus isolates were tested in infectivity assays on GHOST(3) cells (Vödrös et al., 2001). In addition, Env clones from SIVsmB inoculum virus used CXCR4 inefficiently, consistent with the ability of SIVsmB inoculum and day 12 reisolated viruses (H9:12) to infect GHOST(3) cells expressing CXCR4 (Vödrös et al., 2001).

The ability of Env clones to mediate fusion with cells expressing CD4 and a range of other viral coreceptors (not tested in infection assays) was also determined (Fig. 2, and data not shown). Clones from SIVsmB inoculum and reisolates mediated fusion via APJ to a level comparable to GPR15. Again, compared to inoculum and early reisolate clones, the efficiency of APJ use declined over time. Other inoculum and reisolate clones generally exhibited a lesser degree of fusion via APJ. CCR8 supported a low level of fusion for SIVsmB inoculum and day 12 reisolate clones. Later SIVsmB reisolate clones exhibited no or minimal CCR8 use, as did SIVsmA and SIVsmC inoculum and reisolate clones. CCR1, CCR2, CXCR1, and CX3CR1 did not support fusion of any clones examined (data not shown).

In summary, the coreceptor use of Env clones determined in cell-cell fusion assays largely recapitulated results obtained previously with virus isolates. Thus, individual Env clones were found to be multicoreceptor tropic. The SIVsmB inoculum, which was extensively passaged in hPBMCs, and early/rapid progressor SIVsmB reisolate clones generally exhibited a broader coreceptor tropism and more efficient fusion profile on GPR1-, APJ-, CCR8-, and CXCR4-expressing cells than clones from the other inoculum and reisolated viruses, suggesting that passage of the SIVsmB inoculum on human PBMCs resulted in expanded coreceptor tropism. SIVsmB was the only inoculum able to use CXCR4 as a coreceptor. Thus, prolonged passage of this isolate on hPBMCs, known to express a high level of CXCR4 (Bleul et al., 1997), might have selected for CXCR4 using variants. SIVsmA inoculum (grown in the HUT-78 T-cell line, followed by two passages in hPBMCs) and reisolates exhibited similar coreceptor use profiles to SIVsmC inoculum (derived from an infected cynomolgus macaque with only two passages on hPBMCs), indicating that isolation on HUT-78 cells did not skew coreceptor use away from that maintained in vivo.

Utilization of rhesus coreceptors

In general, specific human coreceptors and their macaque equivalents are both able to support infection of SIV. However, some important differences have emerged. For example, rhesus macaque CXCR6 appears to be a less efficient coreceptor for SIVmac strains than human CXCR6 (Pohlmann et al., 2000). Therefore, Env clones from animals MF8 and H9 and the corresponding inoculum viruses (SIVsm A and B, respectively) were tested for fusion capacity via the rhesus counterparts of CCR5, CXCR6, and GPR15 in the presence of human CD4. Results obtained with rhesus CCR5 or GPR15 paralleled those obtained with the human chemokine receptors (Fig. 3A and B), in that all clones that fused with human CCR5 or GPR15 coreceptors were also able to fuse with rhesus CCR5 or GPR15.

Variable CD4 independence of inoculum virus envelopes clones

Some SIV and HIV-2 isolates can mediate CD4-independent infection via CCR5 or CXCR4 (Edinger et al., 1997b, 1999; Endres et al., 1996; Liu et al., 2000; Reeves et al., 1997, 1999; Schenten et al., 1999). We therefore examined whether Env clones from SIVsmA, B, or C inoculum viruses could mediate CD4-independent infection. Relative CD4-independence was determined by assaying Env-mediated fusion of cells expressing CCR5 with or without CD4. Env clones from each of the inoculum viruses differed in their CD4-independent use of CCR5 (Fig. 4). Env clones from SIVsmA, which was isolated on HUT-78 cells from a naturally infected sooty mangabey and passaged twice on human PBMCs, fused with CD4-negative CCR5-expressing cells with 16–27% efficiency compared to CD4/CCR5$^+$ cells. In contrast, prolonged in vitro passage of SIVsmA in human PBMC yielded the SIVsmB inoculum virus from which strictly CD4-dependent clones were obtained. SIVsmC inoculum, derived following in vivo passage of SIVsmA in a cynomolgus macaque, yielded envelopes that widely varied in CD4-independent CCR5 fusion capacity ranging 23–75% efficiency compared to CD4/CCR5$^+$ cells. The differences in CD4-dependence/independence of the inoculum viruses made it particularly interesting to determine whether further evolution of CD4 independence occurred in vivo.

Evolution of CD4 independence in vivo

Envelope clones from reisolated virus obtained from two SIVsmA (MF8 and MF10), two SIVsmB (H9 and H56), and two SIVsmC (H27 and H28) infected cynomolgus macaques were examined for their ability to mediate CD4-independent fusion via CCR5. The majority of envelope clones tested were able to fuse to some extent with CCR5-expressing cells in the absence of CD4 (Fig. 4B). Individual envelope clones from day 12 reisolates from SIVsmC infected animals (H27 and H28) remained divergent in their ability to mediate CD4-independent fusion (Fig. 5D), with some clones exhibiting little CD4 independence while others were able to utilize CCR5 with similar efficiency whether CD4 was present or absent. Env clones from day 12

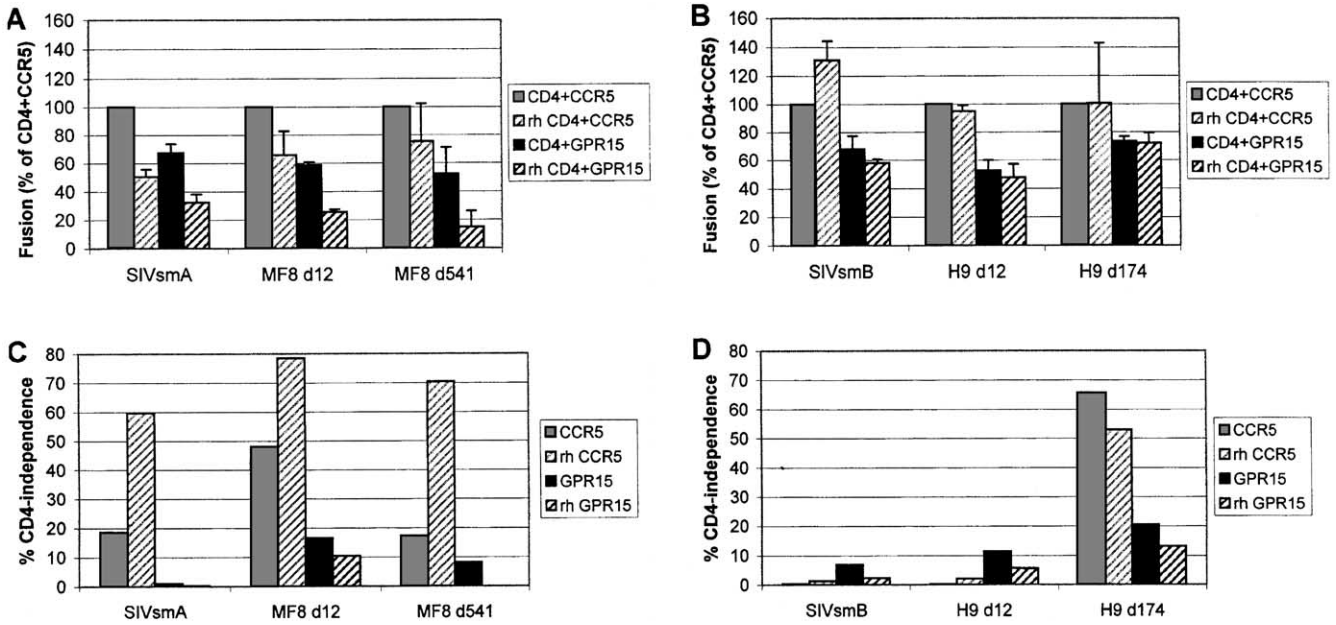

Fig. 3. CD4-dependent and CD4-independent fusion via human and rhesus CCR5 and GPR15. (A and B) Fusion capacity of envelope clones from SIVsmA (MF8) and SIVsmB (H9) inoculated macaques on $CD4^+$ rhesus (rh) and human CCR5- or GPR15-expressing cells, expressed as a percentage of fusion via CD4- and CCR5-expressing cells. (C and D) CD4-independent fusion via human and rhesus CCR5 and GPR15, expressed as a percentage of fusion in the presence of CD4. Error bars represent average value + SD for all clones tested.

reisolates from SIVsmA infected animals exhibited a markedly expanded range of CD4-independence (MF8, 3–84%, MF10, 0–54%, compared to SIVsmA inoculum envelope clones, 16–27%; Fig. 4A). Interestingly, in three of the SIVsmA- and SIVsmC-infected animals (MF8, MF10, and H28), variation in CD4-independent CCR5 use decreased over time (Fig. 4A and B). In contrast, clones from the late isolate of H27 showed a comparably broad range of CD4 independence as did the early clones derived from this animal. This animal did not develop a detectable neutralizing antibody response and died of immunodeficiency within 5 months of SIV infection, whereas MF8, MF10, and H28 developed neutralizing antibodies against both inoculum virus and early reisolates and survived infection for 18 to 20 months (Table 1).

A different pattern of evolution was seen following inoculation with the CD4-dependent SIVsmB virus, with CD4 independence evolving gradually over time (Fig. 4C). Clones from the early reisolate (day 12) of H9 were similarly CD4 dependent in CCR5 use as the inoculum virus. Some degree of CD4-independence evolved in this animal over the course of infection. Four of five clones tested from the late reisolate of H9 used CCR5 alone with 16–31% efficiency, while the fifth clone tested was highly efficient in CD4-independent CCR5 use (153%). An early reisolate was not available from monkey H56; however, clones from 365 and 898 days reisolates showed large variation in CD4-independent CCR5 use, variation being the most pronounced at the later time point (14–158% CD4 independence).

Env clones from MF8, H9, and inoculum viruses (SIVsmA and SIVsmB, respectively) were also tested for the ability to mediate CD4-independent fusion via a range of coreceptors. GPR15 supported CD4-independent fusion of clones derived from both animals (Fig. 3C and D) though the efficiency was on whole reduced compared to CCR5. Envs from MF8 evolved CD4-independent use of GPR15 and enhanced CD4-independence via CCR5 during the first 2 weeks of infection. The efficiency of CD4-independent fusion via both CCR5 and GPR15 decreased by day 541 post infection. In contrast, monkey H9 was inoculated with a virus that was highly dependent on CD4 for CCR5 use and minimally CD4-independent via GPR15. This pattern was maintained in clones from the 12-day reisolate, while late isolates exhibited a marked enhancement of CD4-independent CCR5 fusion and a more modest increase in CD4-independent fusion via GPR15.

MF8 and H9 clones were also examined for CD4-independent use of rhesus CCR5 (98.6% and 100% amino acid homology to human and cynomolgus macaque receptors, respectively) and rhesus GPR15 (97.2% and 99.2% amino acid homology to human and cynomolgus macaque receptors, respectively; Margulies et al., 2001). The pattern of CD4-independent fusion was similar with human and rhesus CCR5 and GPR15 receptors (Fig. 3C and D). Interestingly, rhesus CCR5 supported CD4-independent fusion more efficiently in monkey MF8 than human CCR5. This has been reported previously for SIVmac envelope clones (Edinger et al., 1999; Martin et al., 1997). CD4-independent fusion with GPR15, however, was slightly less efficient with the rhesus receptor compared to the human receptor. In conclusion, the

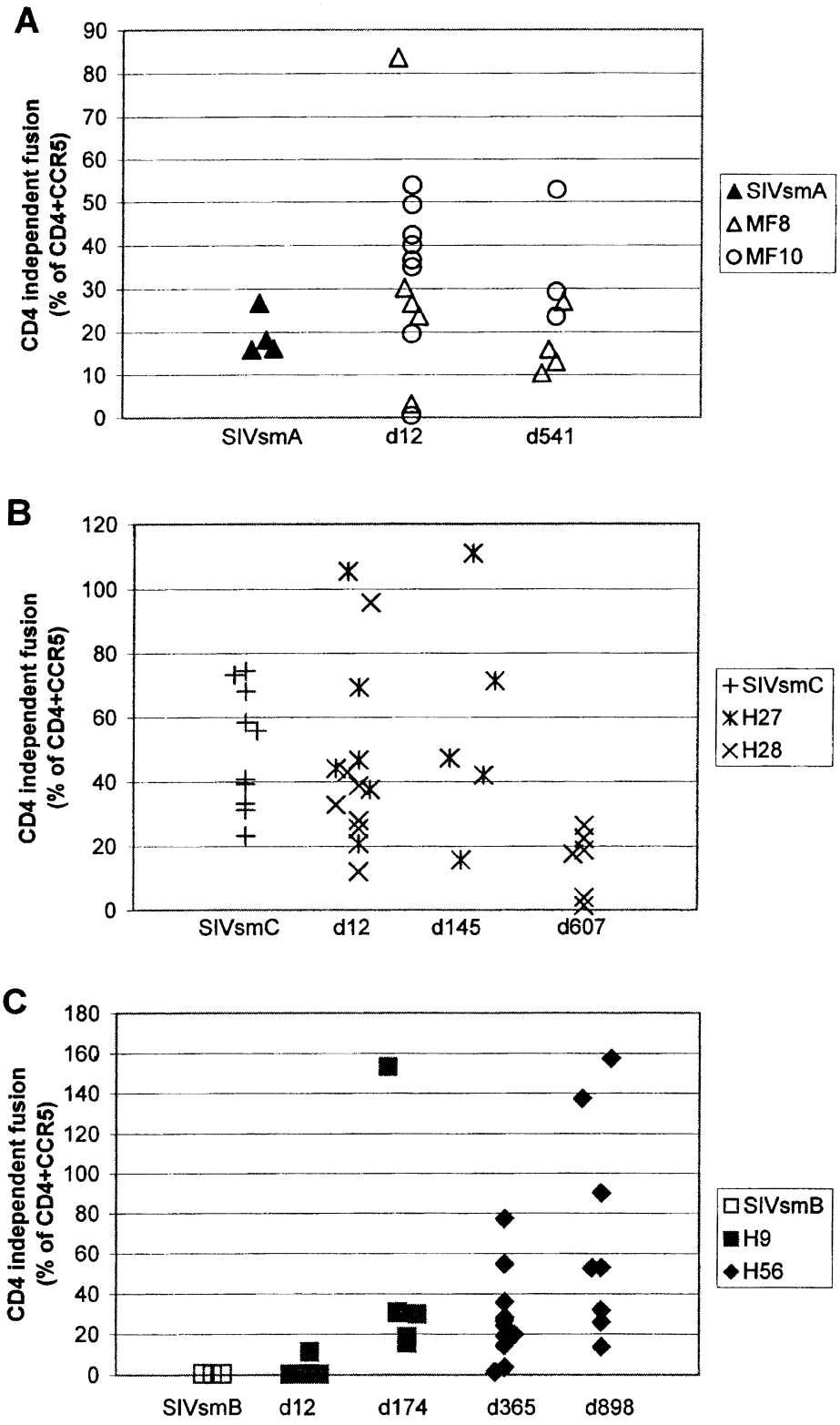

Fig. 4. CD4-independence of inoculum and reisolate virus Env clones from SIVsmA- SIVsmB-, and SIVsmC-infected cynomolgus macaques. CD4-independent fusion via human CCR5 of Env clones derived from animals infected with SIVsmA (A), SIVsmC (B), or SIVsmB (C). Fusion expressed as a percentage of fusion via target cells expressing CD4 and CCR5. Values represent the average of replicates from one to five independent experiments.

in vivo evolution of CD4-independent CCR5 use by SIVsm viruses can vary depending on the properties of inoculum viruses. Furthermore, fusion of SIV envelopes with CCR5 in the absence of CD4 suggests that evolution of CD4 independence may be important for pathogenesis in vivo, either directly or indirectly.

Discussion

Coreceptor use of envelope clones from inoculum viruses and sequential reisolates from SIVsm-infected cynomolgus macaques, with slow or fast disease progression and known neutralizing antibody titers, was evaluated in a cell: cell fusion assay and compared to the ability of corresponding isolates to infect GHOST(3) cells expressing CCR3, CCR5, CXCR4, CXCR6, or GPR15 receptors (Vödrös et al., 2001). The ability of Env clones to mediate fusion via an extended range of coreceptors, including GPR1 and APJ, known to mediate fusion and infection by several SIV isolates and SIV envelope clones (summarized in Broder and Jones-Trower, 1999), was also examined. Our results show that all clones from inoculum viruses, day 12 resiolates, and late reisolates (obtained at the time of immunodeficiency) were able to fuse with varying efficiencies to cells expressing a range of coreceptors that included CCR5, CXCR6, GPR15, GPR1, APJ, and CCR8 in the presence of CD4. The coreceptor use of Env clones reflected the coreceptor specificity of corresponding viral isolates, establishing that these clonally derived envelopes are multicoreceptor tropic. Each envelope has the capacity to mediate fusion via a range of coreceptors that potentially confers a wide cell tropism to the viral isolates.

Some differences between isolates and clonal derivatives were observed. Notably, all clones fused very efficiently with CXCR6-expressing cells, whereas CXCR6-mediated infection of late isolates varied greatly, and three late isolates were not able to infect GHOST(3) cells expressing CXCR6. There are several possible explanations for this difference. First, fusion and infection are different phenomena. It is possible that envelope clones mediate virus attachment to CD4 and CXCR6 resulting in fusion of target and effector cells, whereas they fail to mediate a postentry step that might be important for efficient entry and replication in target cells. This would be supported by our previous findings demonstrating that some late isolates entered GHOST(3) indicator cells expressing CXCR6 (viral Tat-induced GFP expression was detected), but were not able to establish productive infection (measured by viral p27 production in culture supernatants) (Vödrös et al., 2001). Second, differences in receptor expression levels (Platt et al., 1998) or receptor conformation (Baribaud and Doms, 2001; Baribaud et al., 2001) between cells used for fusion and infection may account for varied results. Third, it is also possible that the clones are representative of a minority virus population. However, we feel this is less likely since isolates and clones otherwise exhibit a similar pattern of coreceptor use and there were no major differences in fusion capacity among the different clones derived from the same isolate.

CXCR4 use also differed slightly between clones and isolates. Two viruses [SIVsmB and the day 12 reisolate from macaque H9 (H9:12)] used CXCR4 in the GHOST cell assay. Fusion capacity of corresponding envelope clones with the CXCR4 receptor was less pronounced than infection by the isolates. However, infection of GHOST(3).CXCR4 cells with SIVsmB and H9:12 was only partly inhibited by AMD3100, a specific CXCR4 antagonist, and early isolates could infect the parental GHOST(3) cells (engineered to express CD4 only but known to express low level of endogenous CXCR4) in the presence of AMD3100. Thus we hypothesized that another coreceptor might be expressed on the GHOST(3) cells that can be used by the majority of early isolates (Vödrös et al., 2001). GPR1 is also expressed endogenously on GHOST cells (Soda et al., 1999); however, a consistent pattern of GPR1 use by all Env clones would indicate the presence of a further, as yet unidentified, coreceptor.

Env clones from inoculum viruses with different passage histories were found to exhibit divergent dependence on CD4 for fusion via CCR5. Clones derived from SIVsmA inoculum, isolated on HUT-78 cells from a naturally infected sooty mangabey and passaged twice on hPBMCs, exhibited a relatively narrow range of CD4-independence (16–27%). Further extensive passage on hPBMCs resulted in SIVsmB inoculum from which strictly CD4-dependent clones were derived, suggesting that CD4-dependent viruses may be more fit under these conditions. In vivo passage of SIVsmA in a cynomolgus macaque, followed by reisolation on hPBMCs, resulted in SIVsmC inoculum that gave rise to Env clones exhibiting a broad range of CD4-independence. The typically high level of virus replication observed early after infection can result in a high level of viral diversity. Virions with a phenotype different from the inoculum virus can emerge and replicate. While Env clones from the SIVsmA inoculum virus were relatively homogeneous in CD4-independent CCR5 use, 12 days after infection clonal derivatives of reisolates varied widely in their capacity to fuse with CCR5-expressing cells in the absence of CD4. The high rate of mutations accumulated during virus replication along with depletion of $CD4^+$ T cells at the sites of SIV replication early after infection (Kewenig et al., 1999; Vajdy et al., 2001; Zhou et al., 1999) might select for CD4-independent SIV variants. Variability in CD4-independent CCR5 use decreased over time in three of four SIVsmA- or SIVsmC-infected macaques resulting in an equivalent or lower level of CD4-independent CCR5 use as that seen with inoculum virus clones. These three animals were able to mount neutralizing antibody responses to the inoculum virus and the 12-day reisolate. In contrast, clones from the fourth monkey showed large variation in CD4-independent CCR5 use both early and late and this animal did not mount a neutralizing antibody response. The humoral immune response may thus play a role in controlling CD4-independent virus variants during infection. This is in line with findings of Schmitz et al. (2003) who showed that virus specific antibodies contribute to the control of SIV replication. Recently, Ryzhova et al. (2002) found that CD4-independent envelopes could be isolated from rapid progressor macaques. They found that amino acid changes

at positions 383 and 385 of the SIV envelope distinguished CD4-dependent and independent envelopes. We sequenced three envelope clones from the 12-day isolate of monkey MF8 that differed in CD4-independent use of CCR5 (84%, 30%, or 24%) and found no difference among the clones in these positions. CD4-independent use of CCR5 seems to be coincident with convergent envelope evolution in rapid progressor animals that also lack neutralizing antibodies (Dehghani et al., 2003). We suggest that the lack of neutralizing antibodies may contribute to rapid disease progression and to the evolution of CD4-independent coreceptor use. It will be interesting to compare the neutralization sensitivity of different Env clones obtained from this study. CD4-independent use of coreceptors and neutralization sensitivity of envelopes have been shown to be closely related phenomena (Edwards et al., 2001; Kolchinsky et al., 2001; Means et al., 2001; Puffer et al., 2002). In general, binding of envelope to CD4 leads to conformational changes within gp120 that allow binding to a coreceptor (Kwong et al., 1998; Lapham et al., 1996; Trkola et al., 1996; Wu et al., 1996). Envelopes that bind to coreceptors in a CD4-independent manner are likely to have a "more open" conformation, similar to CD4-triggered CD4-dependent envelopes (Hoffman et al., 1999). Neutralizing antibodies can recognize epitopes in gp120 that becomes exposed after CD4 binding (Javaherian et al., 1994). It is therefore likely that viruses with neutralization-sensitive CD4-independent phenotype are selected against in vivo by the host immune response, in particular by antibodies (Beaumont et al., 2001; Choi et al., 1994; Ye et al., 2000).

Inoculation of SIVsmB into macaques resulted in a different pattern of CD4-independent CCR5 use. Clones from SIVsmB inoculum were strictly CD4-dependent for fusion via CCR5. Clones from the day 12 reisolate from H9 also exhibited a high degree of CD4-dependence. In a few months, however, the virus population became heterogenous and clones appeared that used CCR5 very efficiently in the absence of CD4. This animal was inoculated with a high dose of SIVsm ($>10^5$ MID) and died early of immunodeficiency. Even if there was a strong neutralizing antibody response to the inoculum virus and the day 12 reisolates, the virus recovered at 6 months post infection was resistant to autologous neutralization (Zhang et al., 1993). H56 was inoculated with 1000–10,000-fold less SIVsmB virus. The first clones recovered at day 365 post infection exhibited a broad range of CD4-independence. CD4-independence increased over time with five of eight clones using CCR5 efficiently in the absence of CD4 at day 898. Neutralizing antibody titers against late reisolates were low (Table 1; Zhang et al., 1993).

Our results indicate that there is an initial expansion of viral diversity early after infection that is subsequently controlled either by the immune system or viral selection. Evidence to support this hypothesis is threefold as follows: (1) early but not late viral reisolates exhibit an expanded receptor tropism for the parental GHOST(3) cells (Vödrös et al., 2001); (2) changes in the pattern of CD4-independent CCR5 use of envelope clones derived from sequential isolates; and (3) quality of neutralizing antibody response indicated by increased neutralizing antibody titers against isolates compared to titers against inoculum virus (Table 1). On the whole these points indicate that early after infection there is a rapid change in the virus population, resulting in the emergence of new variants that are relatively CD4-independent and neutralization sensitive, supporting previous studies (Edwards et al., 2001; Kolchinsky et al., 2001; Means et al., 2001; Parren et al., 1999; Puffer et al., 2002). It is likely that expanded tropism, replicative advantage, and sensitivity to neutralization with autologous antibody, alongside cellular immune responses, are selective forces that contribute to establishment of a viral equilibrium during the asymptomatic phase of infection, with increasing viral replication gradually resulting in immunodeficiency and less viral control. Selection of CD4-independent SIVsm variants early after infection with SIVsmA may play a significant role in promoting virus replication, though these viruses appear to be controlled later after infection, perhaps because CD4-independence has thus far been invariably associated with enhanced sensitivity to neutralizing antibodies. Evolution to CD4-independence/dependence in conjunction with the humoral immune response likely shape the course of SIV pathogenesis.

Materials and methods

Animals and viruses

The SIVsm isolate SMM-3 (kindly provided by P. Fultz and H. McClure, Yerkes Regional Primate Research Center, Atlanta, GA) originated from a naturally infected sooty mangabey (*Cercocebus atys*) (Fultz et al., 1986). This virus was isolated and passaged twice in the human T-cell line HUT-78, followed by two passages in human peripheral blood mononuclear cells (hPBMC) (Putkonen et al., 1989). Virus derived from cell-free supernatant of hPBMC cultures, harvested 8 days after infection, was the source of inoculum A. Inoculum B was derived by further passage of inoculum A on hPBMC for 3 months. Inoculum C was derived from a cynomolgus macaque that had been infected with inoculum A and developed diarrhea, weight loss, and tachypnea 245 days post inoculation. Virus was reisolated from macaque PBMCs by cocultivation with hPBMC and the cell-free supernatant of this coculture was used as inoculum C.

All three inoculum viruses used CCR5, CXCR6, and GPR15 as coreceptors for infection of $CD4^+$ GHOST(3) indicator cell lines (Vödrös et al., 2001). In addition, SIVsmB was also able to use CXCR4 (Vödrös et al., 2001). Eight cynomolgus macaques were infected intravenously with SIVsmA (×4), SIVsmB (×2), or SIVsmC (×2) isolates (Putkonen et al., 1989; Zhang et al., 1993) and the

coreceptor use of reisolated viruses examined (Vödrös et al., 2001). Coreceptor use of day 12 reisolates was similar to that of inoculum viruses (Vödrös et al., 2001) (Table 1). CCR5 use remained stable in later reisolates while the capacity to use other coreceptors decreased over time (Vödrös et al., 2001).

Sera from seven SIVsm-infected macaques exhibited neutralizing activity (Zhang et al., 1993; Table 1). Sera obtained at specific time points post infection could neutralize previous but not current or subsequent isolates, with the exception of macaque H27 that did not mount any neutralizing antibody response. Sera obtained from animals with the latest onset of immunodeficiency (H28 and H56) appeared to have the broadest neutralizing activity, since they could neutralize virus isolates derived from other SIVsm-infected macaques (Zhang et al., 1993).

Amplification and cloning of SIV envelopes

Viral envelope genes were cloned from inoculum viruses and reisolates obtained from six cynomolgus macaques inoculated with SIVsmA (×2), SIVsmB (×2), or SIVsmC (×2) as follows: Human PBMCs were infected with inoculum or reisolated viruses, 7 days post infection, cytopathicity was apparent and genomic DNA was isolated from infected cells with a Qiagen blood and cell culture DNA kit; 250, 50, or 10 ng of genomic DNA was used as a template for PCR amplification of envelopes using SIVsmFI (5′ CTAATAACGCGTCTGCATCAGACAAGTAAGTATG 3′) and SIVsmRI (5′ CATCAGCGGCCGCATCTACATCATCCATGTTTTGTT 3′) oligonucleotides. Two to four independently amplified PCR products were cut with *Mlu*I and *Not*I restriction enzymes (restriction sites in oligonucleotides) and envelope genes ligated into the *MluI/Psp*OMI-restricted pCI-PRE expression vector (kindly provided by Ted C. Pierson). Four or five clones were selected from each ligated PCR product and screened by restriction digestion to determine whether plasmids contained Env gene inserts of the expected size. The functionality of Env gene clones was determined in a cell-cell fusion assay, as described below.

Cell-cell fusion assay

QT6 “effector” cells, transfected with Env expression plasmids and infected with a vaccinia virus encoding T7 polymerase (vTF1.1) (Alexander et al., 1992), were added to QT6 “target” cells cotransfected with a luciferase reporter construct under the control of a T7 promotor (T7-luc), CD4, and coreceptor or pcDNA3.1 (control) expression plasmids. Cell:cell fusion, resulting from a functional interaction between Env-expressing effector cells and receptor-expressing target cells, was detected by assaying for T7 polymerase-driven luciferase expression. This assay has been described in detail previously (Rucker et al., 1997).

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