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# Enhanced CD4+ cellular apoptosis by CCR5-restricted HIV-1 envelope glycoprotein variants from patients with progressive HIV-1 infection

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ABSTRACT

CCR5-using (R5) human immunodeficiency virus type 1 (HIV-1) strains cause CD4+ T-cell loss in most infected individuals, but mechanisms underlying cytopathicity of R5 viruses are poorly understood. We investigated mechanisms contributing to R5 envelope glycoprotein (Env)-mediated cellular apoptosis by constructing a panel of retroviral vectors engineered to co-express GFP and R5 Envs derived from two HIV-1-infected subjects spanning asymptomatic (Early, E-R5 Envs) to late stages of infection (Late, L-R5 Envs). The L-R5 Envs induced significantly more cellular apoptosis than E-R5 Envs, but only in Env-expressing (GFP-positive) cells, and only in cells where CD4 and CCR5 levels were limiting. Studies with fusion-defective Env mutants showed induction of apoptosis required membrane-fusing events. Our results provide evidence for an intracellular mechanism of R5 Env-induced apoptosis of CD4+ cells that requires membrane fusion. Furthermore, they contribute to a better understanding of mechanisms involved in CD4+ T-cell loss in subjects experiencing progressive R5 HIV-1 infection.



## Introduction

Progression of human immunodeficiency virus type 1 (HIV-1) infection from early, asymptomatic stages of disease to acquired immunodeficiency syndrome (AIDS) is associated with a switch in viral coreceptor specificity from CCR5—using (R5) viral strains to those able to use CXCR4 (X4) or both coreceptors (R5X4) in 40–50% of infected adults (Bjorndal et al., 1997; Connor et al., 1997; Karlsson et al., 1994a; Koot et al., 1993; Tersmette et al., 1989) [reviewed in (de Roda Husman and Schuitemaker, 1998)]. However, X4 or R5X4 variants are absent in 50–60% of HIV-1 infected individuals who progress to AIDS (de Roda Husman et al., 1999; Jansson et al., 1999; Jansson et al., 1996; Karlsson et al., 2004; Koning et al., 2003) [reviewed in (Gorry et al., 2004)]. Therefore, the persistence of an exclusive R5 viral population *in vivo* is sufficient to cause immunodeficiency in the majority of HIV-1 infected individuals who progress to AIDS.

Whilst much is known about the molecular basis of pathogenicity of late-emerging X4 and R5X4 viruses (Glushakova et al., 1995, 1998; Picchio et al., 1998) [reviewed in (de Roda Husman and Schuitemaker, 1998)], the molecular mechanisms contributing to pathogenicity of R5 HIV-1 strains are not entirely understood (Gorry et al., 2004). R5 viruses are intrinsically cytopathic, but exert pathogenic effects that are distinct from those of X4 or R5X4 viruses (Fais et al., 1999; Grivel and Margolis, 1999; Harouse et al., 1999). Compared to R5 HIV-1 strains isolated from asymptomatic individuals (referred to hereafter as “Early” or E-R5 viruses), R5 HIV-1 strains isolated from patients with late stages of infection (referred to hereafter as “Late” or L-R5 viruses) have been reported to have enhanced macrophage-tropism (Gray et al., 2005; Li et al., 1999; Tuttle et al., 2002), increased viral fitness (Borggren et al., 2008; Repits et al., 2005, 2008), increased ability to scavenge low cellular levels of CD4 and CCR5 for HIV-1 entry (Gray et al., 2005), reduced sensitivity to HIV-1 entry inhibitors (Gray et al., 2005; Repits et al., 2005) [reviewed in (Sterjovski et al., 2006)] and RANTES (Jansson et al., 1999; Karlsson et al., 2004; Koning et al., 2003), and increased sensitivity to the Env mAb IgG1b12 (Gray et al., 2005). Decreased RANTES sensitivity has been attributed to an increased flexibility of the R5 envelope glycoproteins (Env) that subsequently alters the

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mode and efficiency of CCR5 binding (Karlsson et al., 2004). Further studies of R5 Envs cloned from E-R5 and L-R5 viruses showed L-R5 Envs have enhanced CCR5-dependent fusogenicity and faster HIV-1 entry kinetics, and are more likely to exist in conformations that promote greater exposure or stabilization of the CD4 binding site (CD4bs) in gp120 (Sterjovski et al., 2007). Together, these findings provide evidence that L-R5 viruses have intrinsic properties distinguishing them from E-R5 viruses which may enhance their cytopathic effects, and that these properties are likely to be linked to Env conformations that enhance CD4 and/or CCR5 interactions.

In addition to receptor binding and mediating HIV-1 entry, Env is responsible for the cytopathic effects of HIV-1 infection in cultured cells (Sodroski et al., 1986), and may contribute to death of uninfected cells through bystander mechanisms (Holm et al., 2004). Furthermore, passage of chimeric simian-HIV (SHIV) strains in macaques demonstrated enhancement of pathogenicity that was associated with mutations in Env (Cayabyab et al., 1999; Karlsson et al., 1997, 1998; Liu et al., 1999; Stephens et al., 1996; Stephens et al., 1997), which often resulted in increased Env-mediated cytopathicity (Etemad-Moghadam et al., 2000, 2001; Karlsson et al., 1998; Liu et al., 1999; Si et al., 2004). Together, these studies suggest that alterations in the HIV-1 Env which augment cytopathicity may contribute to CD4+ T-cell loss during progressive HIV-1 infection.

To determine whether Env mediated cytopathicity increases during progressive R5 HIV-1 infection, we characterized E-R5 and L-R5 Envs cloned from sequential viruses isolated from two individuals who experienced progressive HIV-1 infection whilst harboring R5 HIV-1 variants. Our results show that L-R5 Envs from both subjects induced significantly more cellular apoptosis than E-R5 Envs when delivered intracellularly by retroviral transduction, but only in cells where CD4 and CCR5 levels were limiting, and only in Env-expressing cells. Env-mediated bystander apoptosis was not evident in this system. These results provide evidence for an intracellular mechanism of R5 Env-induced apoptotic cell death requiring membrane fusion which, in some individuals may contribute to CD4+ T-cell loss during progressive R5 HIV-1 infection.

## Results

### *Biological activities of primary R5 HIV-1 Env clones*

To better understand Env determinants involved in progressive R5 HIV-1 infection, we produced a longitudinal panel of E-R5 and L-R5 Envs cloned from two subjects, IK1 and IK2, spanning asymptomatic through to late stage HIV-1 infection. The Envs from subject IK1 used in this study, IK1-E-1 and -2 and IK1-L-1 and -2, have been reported previously (Sterjovski et al., 2007) and were confirmed to be functional and of R5 phenotype in single-round HIV-1 entry assays and fusion assays (Table 1), and shown to process gp120 efficiently from gp160 in transfected cells by Western blotting (Sterjovski et al., 2007). In order to expand this set of longitudinal R5 Envs to include those from another subject, Envs were cloned from E-R5 and L-R5 viruses isolated from subject IK2 (Karlsson et al., 2004) into the pSVIIIEnv expression vector. Two independent, functional and properly processed Envs cloned from each virus were identified by single round entry assays in JC53 cells using Env-pseudotyped GFP reporter viruses, fusion assays, and Western blotting (Table 1, and data not shown). To confirm the coreceptor specificity of the cloned Envs, Env-pseudotyped GFP reporter viruses were used in single round entry assays with Cf2th cell lines stably expressing CD4/CCR5 or CD4/CXCR4 (Table 1). The X4 HXB2, R5X4 89.6 and R5 ADA, JRCSF and YU2 Envs were used as positive controls. A non-functional Env, ΔKS Env, was used as a negative control to determine background levels of GFP expression. All Envs from subjects IK1 and IK2 used CCR5 for HIV-1 entry, similar to the coreceptor specificity of the primary isolates from which they were cloned. Thus, we established and validated a longitudinal panel of eight functional E-R5 and L-R5 Envs derived from two subjects with progressive R5 HIV-1 infection.

**Table 1**
Characteristics of primary R5 viruses and Env clones.

| Virus[a] | Description[b] | Env clone[c] | Coreceptor usage[d] | |
|---|---|---|---|---|
| | | | CCR5 | CXCR4 |
| IK1-E | E-R5 | IK1-E-1 | ++ | − |
| | | IK1-E-2 | + | − |
| IK1-L | L-R5 | IK1-L-1 | ++ | − |
| | | IK1-L-2 | +++ | − |
| IK2-E | E-R5 | IK2-E-1 | + | − |
| | | IK2-E-2 | ++ | − |
| IK2-L | L-R5 | IK2-L-1 | ++ | − |
| | | IK2-L-2 | +++ | − |
| | | Control Envs | | |
| | | ΔKS Env | − | − |
| | | HXB2 Env | − | +++ |
| | | 89.6 Env | +++ | +++ |
| | | ADA Env | +++ | − |
| | | JRCSF Env | +++ | − |
| | | YU2 Env | +++ | − |

[a] The phenotypes of the primary R5 HIV-1 isolates, and clinical characteristics of the subjects from whom they were isolated have been described in detail previously (Jansson et al., 1999; Karlsson et al., 1994b, 2003, 2004).
[b] E-R5, Early R5 HIV-1 isolate; L-R5, Late stage R5 HIV-1 isolate.
[c] Functional Env clones were identified by infection of JC53 cells with Env-pseudotyped GFP reporter viruses and by fusion assays, as described in Materials and Methods (data not shown).
[d] Coreceptor usage of functional Envs was determined by infection of Cf2-CD4/CCR5 and Cf2-CD4/CXCR4 cell lines with Env-pseudotyped GFP-reporter viruses, as described in Materials and methods. The coreceptor usage of Envs derived from viruses IK1-E and IK1-L has been described previously (Sterjovski et al., 2007). GFP positive cells were counted manually by fluorescence microscopy and scored as − (no GFP positive cells), +/− ( 1 to 5% GFP positive cells),+ (5 to 10% GFP positive cells), ++ (10 to 30% GFP positive cells), or +++ (>30% GFP positive cells).

### *Utility of GFP expression from psrHIVenvGFP vectors as a surrogate for Env expression in transduced cells*

Previous studies showed that Env could be detected in every GFP-positive (GFP+) cell following transduction of cell cultures with the X4 HXB2 Env using recombinant viruses encoding psrHIVenvGFP vectors (LaBonte et al., 2000), indicating that in this retroviral transduction system GFP can be used as a simple, non-disruptive and reliable way to determine which cells in a transduced population are and are not expressing Env. Nonetheless, we wished to validate the reliability of GFP as a surrogate for R5, X4 and R5X4 Env expression, and to confirm whether Envs with these phenotypes are correctly processed in transduced cells. JC53 cells were transduced with equivalent titers of replication-defective viral vectors expressing X4 HXB2, R5X4 89.6 or R5 ADA Envs, or the ΔEnv mutant as a negative control, and 48 h later were fixed and permeabilized, and examined for GFP and Env expression using fluorescence microscopy (Fig. 1A). In the cultures transduced with X4, R5X4 or R5 Envs, Env was detected in every GFP+ cell and vice versa. In JC53 cells transduced to express the ΔEnv mutant, many GFP+ cells were present but none stained with the primary and secondary antibodies used to detect Env, indicating their specificity for Env. Further experiments comparing permeabilized to non-permeabilized cells showed that the vast majority of detectable Env staining was intracellular, and the small amounts of cell surface-detectable Env were evident in only a proportion of GFP+ cells (Supplementary Fig. 1). Consistent with this observation, the majority of the GFP+ cells remained as single cells (Fig. 1A and Supplementary Fig. 1), although some syncytia were evident. Transduced JC53 cells were also lysed and analyzed for gp160/gp120 expression by Western blotting at 48 h post-transduction (Fig. 1B). In the cultures transduced with X4, R5X4 or R5 Envs, gp120 bands were detected indicating correct processing of gp160 to gp120. Thus, we confirmed that GFP expression is a reliable

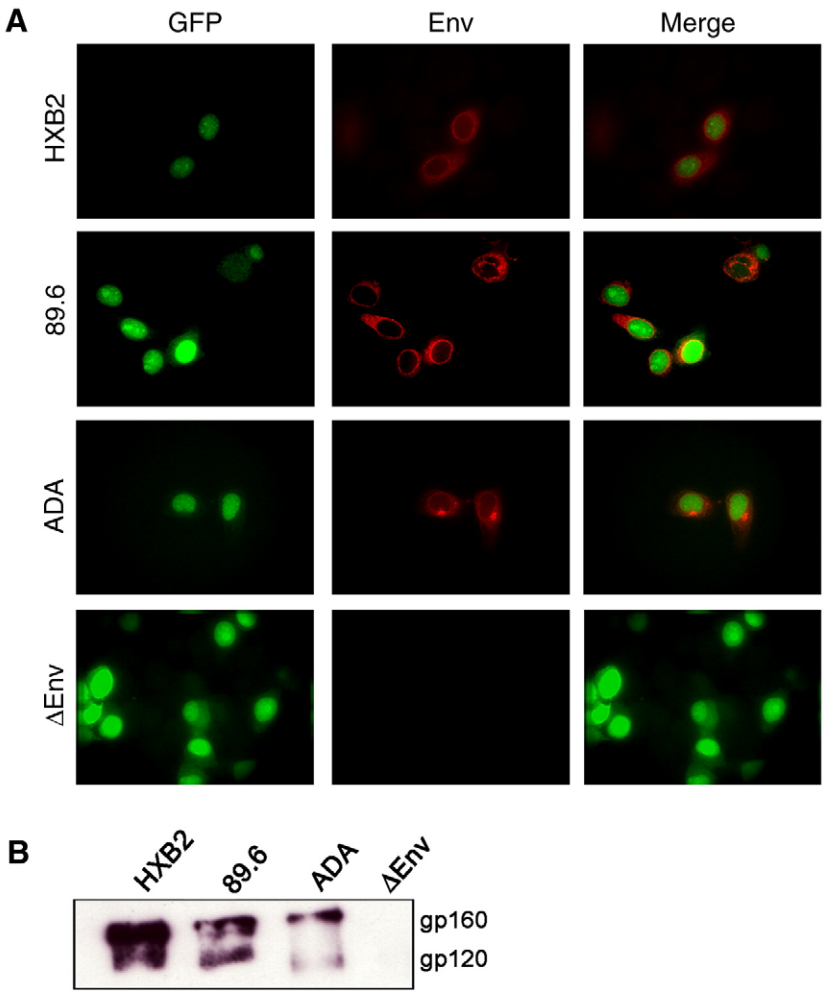


**Fig. 1.** Coexpression of GFP and Env in transduced cells and gp120 production. (A) JC53 cells were transduced with psrHIVenvGFP vectors encoding HXB2, 89.6 or ADA Env, or with psrHIVΔenvGFP. At 48 h post-transduction cells were fixed and permeabilized, labeled with the human polyclonal antibody BB10 that is strongly reactive for Env protein but is not reactive against HIV-1 regulatory/accessory proteins (Gorry et al., 1998, 1999) and Texas Red-conjugated anti-human antibody. Cells were analyzed for GFP (left panel) or Env expression (middle panel). The merged images (right panel) show that every GFP-expressing cell also expresses HXB2, 89.6 or ADA Env protein. Note that in cells transduced with the psrHIVΔenvGFP vector, GFP-expressing cells are present but they do not stain with the primary and secondary antibodies, indicating the specificity of these antibodies for Env in cells transduced with psrHIVenvGFP vectors. Each image is one z section from the deconvolved z-stack image. The micrographs shown are representative of 5 random fields of one experiment and are of ×600 final magnification. (B) Env expression in lysates of transduced JC53 cells was measured by Western blot analysis using rabbit anti-gp120 polyclonal antisera, as described in Materials and methods. The positions of gp160 and gp120 are indicated on the right.

surrogate for correctly processed gp120 of diverse phenotypes when expressed from psrHIVenvGFP vectors, and that in this system Env expression is predominantly intracellular.

*Direct and bystander cellular apoptosis by prototypic R5 Envs*

Previous studies have characterized cytolysis induced by the R5 ADA Env in cells after transduction with psrHIVenvGFP vectors using non-specific measures such as cell viability assays and syncytia formation (LaBonte, Madani, and Sodroski, 2003), but whether cytolysis from intracellular ADA Env expression was due to apoptosis or other mechanisms, or whether Env-mediated cytolysis is a phenotype of other R5 Envs, is unknown. To determine whether ADA and other prototypic R5 Envs cause apoptotic cell death following transduction with psrHIVenvGFP vectors, JC53 and PM1 cells were transduced with vectors expressing the R5 ADA, JRCSF or YU2 Envs. Cells transduced with the ΔEnv construct were included to control for the effects of non-Env elements in the transducing viruses. JC53 and PM1 cells were used because they are cell lines that express both CD4 and CCR5 and are permissive to R5 HIV-1 infection. Cells were harvested at 24, 48, 72 and 96 h post-transduction and stained for active caspase-3 expression, which was quantified separately by flow cytometry in GFP+(i.e., Env-expressing) and GFP-negative (GFP-) (i.e., Env-negative) cell populations to determine the level of direct and bystander cell apoptosis, respectively (Fig. 2). The total cell numbers, individual transduction percentages, and total numbers of GFP+ and GFP-cells analyzed in these and subsequent experiments are summarized in Supplementary Table 1. Representative flow cytometry data from which Fig. 2 was derived are shown in Supplementary Fig. 2. In JC53 and PM1 cells, each of the R5 Envs induced apoptosis in more than 80% or 40% of GFP+ cells by 96 h post-transduction, respectively. This was comparable to the levels of apoptosis mediated by the X4 HXB2 and R5X4 89.6 Envs in GFP+ populations of both cell types (data not shown). Background levels of apoptosis that did not exceed 10% of GFP+ cells were observed in cells transduced with the ΔEnv construct, indicating that the increases in apoptosis observed were due to Env. In both cell types, cellular apoptosis in GFP-cell populations was not measured above background levels, indicating that bystander apoptosis was not evident in this system. These results indicate that diverse R5 Envs may cause significant levels of apoptosis in Env expressing cells, similar to X4 and R5X4 Envs, and that JC53 cells are more permissive to Env-mediated apoptosis than PM1 cells.

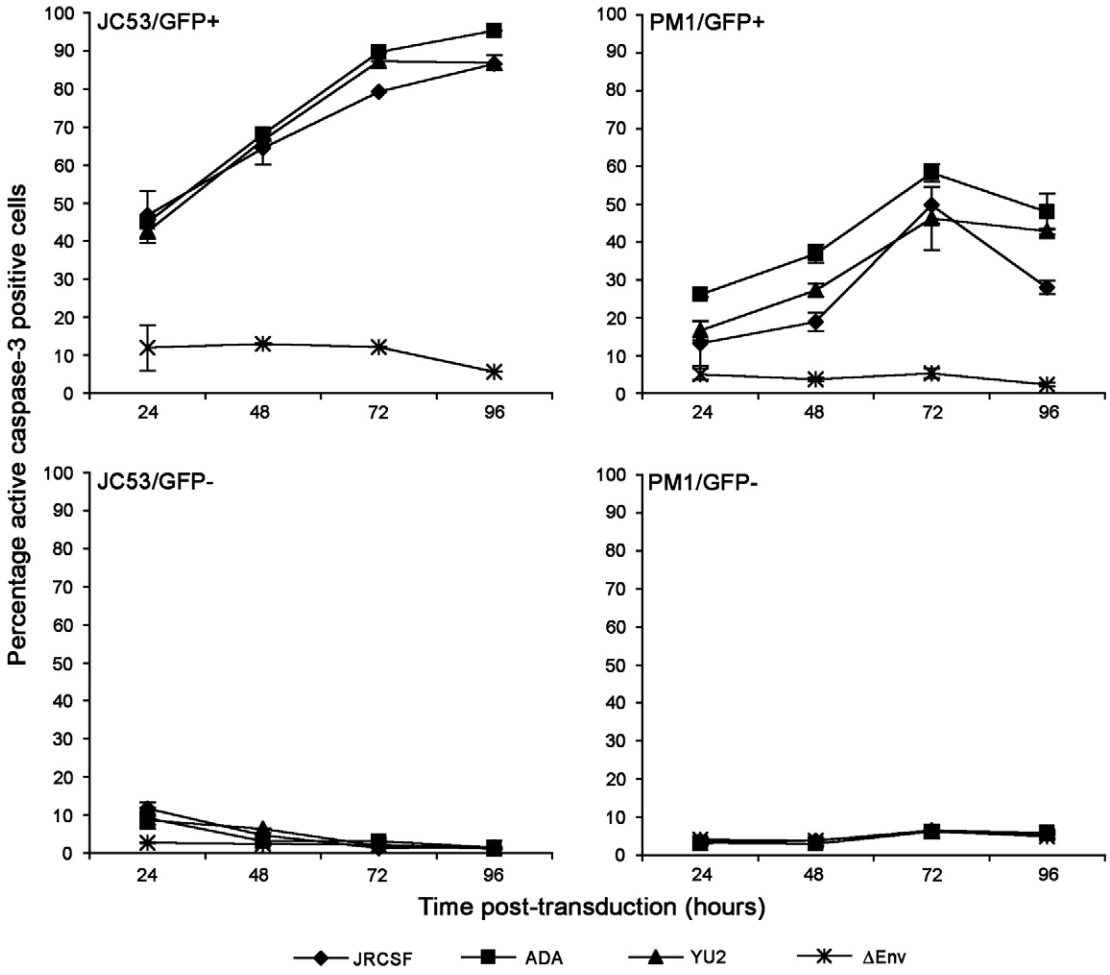


**Fig. 2.** Direct and bystander cellular apoptosis by prototypic R5 Envs. JC53 (left panels) or PM1 cells (right panels) were transduced with psrHIVenvGFP vectors encoding R5 JRCSF, ADA or YU2 Envs, or with psrHIVΔenvGFP as a negative control as described in Materials and methods. At the indicated times after transduction, cells from independent duplicate cultures were harvested, fixed and permeabilized, and labeled with an anti-human active caspase-3 antibody and PE-conjugated anti-rabbit IgG as described in Materials and methods. The percentage of GFP+(Env-expressing) and GFP-(Env-negative) cells that stained positive for active caspase-3 expression was determined by flow cytometry. The data shown are means of duplicate wells, and the error bars represent standard deviations.

*Membrane fusing activity is required for apoptosis of Env-expressing cells*

To better understand the molecular mechanisms involved in activation of the caspase-3 pathway in Env-expressing cells, PM1 cells were transduced with vectors expressing the R5 ADA, X4 HXB2 or R5X4 89.6 Envs in parallel with fusion-defective F/Y mutants (Fig. 3). Compared to the parental Envs, these fusion-defective mutants express equivalent amounts of properly processed gp120 and retain the ability to bind CD4 and coreceptor, but they cannot mediate membrane fusion (LaBonte et al., 2000, 2003) (and data not shown). Although apoptosis was induced in the majority of GFP+ cells expressing ADA, HXB2 or 89.6 Envs by 72 h post-transduction, background levels of apoptosis were observed in cells expressing the respective fusion-defective Env mutants. Thus, activation of the caspase-3 pathway by Env is dependent on membrane fusing activity, and this appears to be a common requirement of Envs with diverse phenotypes.

*Direct and bystander cellular apoptosis by primary E-R5 and L-R5 Envs*

To determine whether primary R5 Envs isolated from individuals with asymptomatic or late stages of HIV-1 infection differ in their ability to induce cellular apoptosis, the *Kpn*I-to-*Bam*HI region of pSVIII Envs encoding E-R5 and L-R5 Envs of subjects IK1 and IK2 (Table 1) was cloned into psrHIVenvGFP vectors, and equivalent titers of recombinant virus was used to transduce JC53 and PM1 cells (Fig. 4). In JC53 cells, all of the primary R5 Envs induced apoptosis in more than 90% of GFP+ cells by 96 h post-transduction. There was no accumulation of apoptosis in GFP-cells, indicating that apoptosis was confined to Env-expressing cells. In both subjects, there was no difference in the levels of cellular apoptosis in JC53 cells between E-R5 and L-R5 Envs. In PM1 cells, the L-R5 Envs from both subjects induced apoptosis in 40 to 60% of GFP+ cells at 72 h post-transduction which decreased marginally by 96 h, whereas the E-R5 Envs from both subjects induced apoptosis in only 10 to 25% of GFP+ cells. There was no accumulation of apoptosis in GFP-cells, similar to the results using JC53 cells. The overall levels of Env expression and gp120 processing by the E-R5 and L-R5 Envs were similar (data not shown). These results indicate that, in both subjects, L-R5 Envs induce greater levels of apoptosis than E-R5 Envs when expressed in PM1 cells, but not when expressed in JC53 cells.

*Sequence analysis*

To better understand Env determinants contributing to the increased ability of L-R5 Envs to induce apoptosis in PM1 cells, the unique *Kpn*I-to-*Bam*HI region of gp160 was sequenced and analyzed

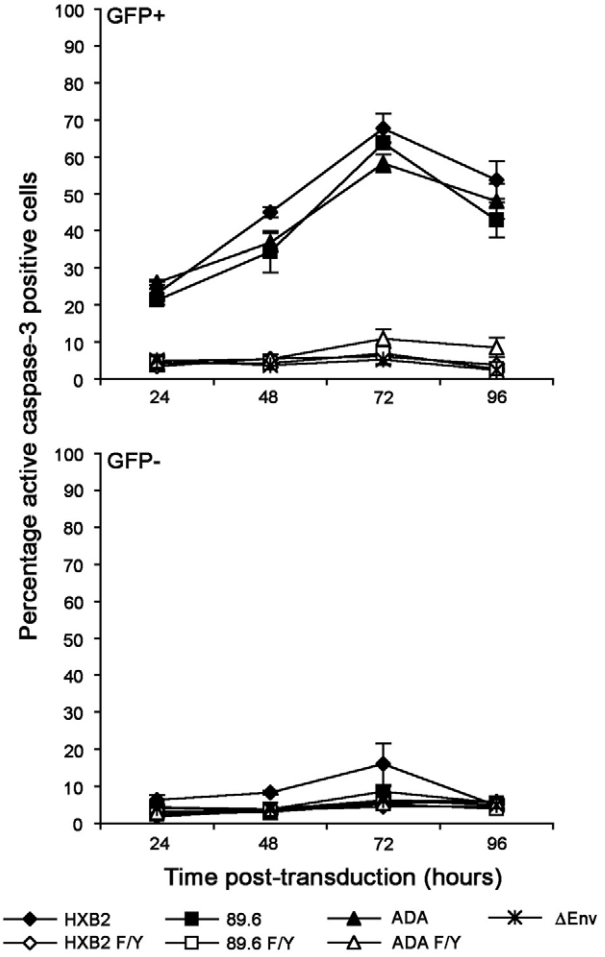


**Fig. 3.** The requirement of membrane fusion for cellular apoptosis by diverse Envs. PM1 cells were transduced with psrHIVenvGFP vectors encoding HXB2, 89.6 or ADA Envs in parallel with their respective fusion-defective F/Y mutants, or with psrHIVΔenvGFP as a negative control as described in Materials and methods. At the indicated times after transduction, cells from independent duplicate cultures were harvested, fixed and permeabilized, and labeled with an anti-human active caspase-3 antibody and PE-conjugated anti-rabbit IgG as described in Materials and methods. The percentage of GFP+(Env-expressing) and GFP-(Env-negative) cells that stained positive for active caspase-3 expression was determined by flow cytometry. The data shown are means of duplicate wells, and the error bars represent standard deviations.

for amino acid changes which may influence Env function. The E-R5 and L-R5 Envs could not be segregated based on the total number of potential N-linked glycosylation sites (PNGS) in gp120 and gp41, nor when numbers of PNGS were stratified across the V1V2, V4 or V5 regions of gp120 (data not shown). The net charge of the V3 region of gp120 was+3 or+4, which is typical of HIV-1 subtype B R5 Envs (Briggs et al., 2000; Milich et al., 1993), and V3 charge did not segregate E-R5 from L-R5 Envs. There was no clear signature pattern that unambiguously segregated E-R5 from L-R5 Envs that was common to both subjects, suggesting that Env sequence alterations contributing to enhancement of apoptosis in PM1 cells are likely to be patient-specific and/or context dependent. There were numerous patient-specific single amino acid changes which segregated E-R5 from L-R5 Envs that could potentially influence Env function (see Supplementary Table 2). These include conserved changes in the C1, V1, V2, C2, V3, C3, V4 and V5 region of gp120, some of which resulted in alterations in glycosylation patterns or alterations in the CD4bs, and within gp41. Further studies are required to better understand the significance of these amino acid alterations in modulating the ability of R5 Envs to cause apoptosis in PM1 cells.

## Discussion

In this study, we generated and characterized a panel of E-R5 and L-R5 Envs cloned from sequential viruses isolated from two individuals who experienced progressive HIV-1 infection whilst harboring R5 HIV-1 variants. Our results show that L-R5 Envs from both subjects induced greater levels of cellular apoptosis that E-R5 Envs when delivered intracellularly by retroviral transduction, but only in PM1 cells where CD4 levels were limiting, and only in Env-expressing cells. The expression levels of CD4 and CCR5 in PM1 cells are similar to those in primary CD4+ T-cells, whereas JC53 cells over-express both receptors (data not shown). Thus, expression of Env in PM1 cells from psrHIVenvGFP vectors is a valid model for studying the activity of Env expressed during HIV-1 infection in CD4+ cells.

Our studies with fusion-defective F/Y Env mutants indicate that binding of gp120 to CD4 and coreceptor is not sufficient to activate caspase-3 dependent apoptosis in Env-expressing cells. The ability to induce apoptosis was dependent on the membrane fusing activity of the Envs. These results support and extend previous studies that examined the role of F/Y Env mutants on Env-mediated cytopathicity using non-specific measures of cell viability (LaBonte et al., 2000, 2003). Passage of SHIV strains in macaques demonstrated enhancement of pathogenicity that was associated with Env mutations which enhanced fusogenicity (Etemad-Moghadam et al., 2001, 2000; Karlsson et al., 1998; Liu et al., 1999; Si et al., 2004), suggesting that increased membrane fusing activity of Env contributes to viral pathogenicity in this animal model. In addition, compared to E-R5 HIV-1 Envs, the L-R5 Envs from subject IK1 as well as those from a cross-sectional cohort of individuals have enhanced CCR5-mediated fusogenicity (Sterjovski et al., 2007), suggesting that enhanced membrane fusing activity of Env may contribute to loss of CD4+ T-cells in subjects with progressive R5 HIV-1 infection. Furthermore, in the cross-sectional study of L-R5 Envs, enhanced fusogenicity resulted from enhanced exposure or stabilization of the CD4bs in gp120 (Sterjovski et al., 2007). Because the retroviral constructs used in the present study were designed to express Env in a similar context to that from the native HIV-1 RNA, the levels of Env expressed are similar to those in HIV-1 infected cells, and expression of Env is predominantly intracellular (LaBonte et al., 2000). Thus, in our studies the induction of apoptosis seems to occur through an intracellular mechanism. Taken together, these studies suggest that in PM1 cells, L-R5 Envs may have an increased ability to interact with relatively low intracellular pools of CD4 than E-R5 Envs, with subsequent fusion and disruption of internal membranes leading to apoptosis. The reliance on Env-mediated fusion suggests a putative role of gp41 in this process. Further studies with cells engineered to express different levels of CD4 are required to test the hypothesis that Env-mediated apoptosis of Env-expressing cells occurs through a CD4-dependent mechanism.

However, our results are inconsistent with those of a previous study, which showed that higher levels of CD4 expression protected cells from Env-mediated cell death (LaBonte et al., 2003). In this study, high CD4 levels expressed in the Cf2th canine thymocyte cell line sequestered Env intracellularly and abrogated processing of gp160 to gp120 and hence, reduced Env-mediated cell death. In our study JC53 cells, which express higher levels of CD4 than the CD4-expressing Cf2th cell line used in the previous study, were highly sensitive to Env-mediated apoptosis, even more so than PM1 cells which expressed much lower levels of CD4. In addition, gp120 was correctly processed when expressed from the psrHIVenvGFP vectors in JC53 cells (Fig. 1B). Thus, high CD4 levels did not prevent Env-mediated apoptosis in our study. Biological differences between the human cell lines used in our study and the canine cell lines used previously (LaBonte et al., 2003), and differences between the assays used to measure cell death (active caspase-3 staining versus cell viability and syncytia formation) may explain these discrepancies.

In cells transduced with psrHIVenvGFP vectors, cellular apoptosis occurred only in Env-expressing cells and not in bystander cells. This

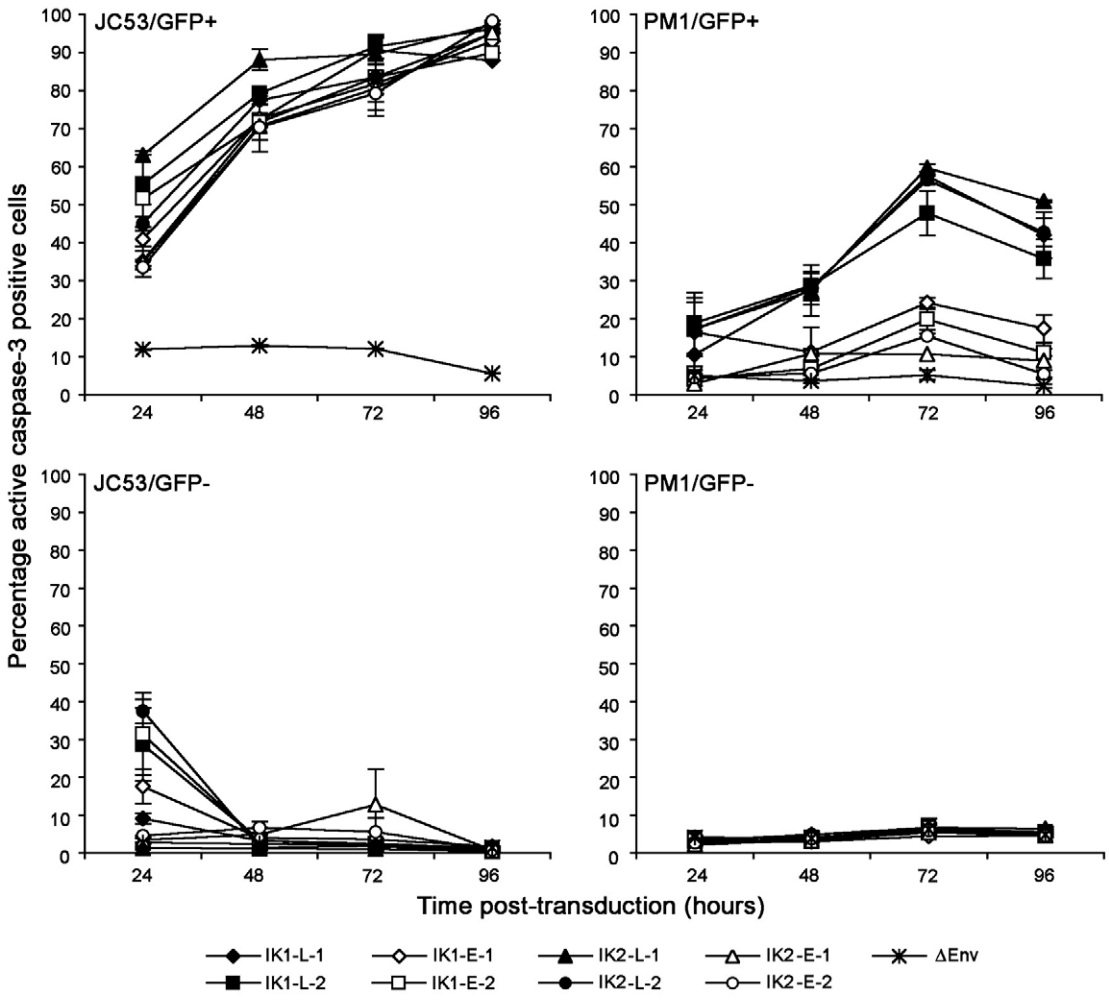


**Fig. 4.** Direct and bystander cellular apoptosis by primary E-R5 and L-R5 Envs. JC53 (left panels) or PM1 cells (right panels) were transduced with psrHIVenvGFP vectors encoding primary E-R5 or L-R5 Envs derived from subjects IK1 and IK2, or with psrHIVΔenvGFP as a negative control as described in Materials and methods. At the indicated times after transduction, cells from independent duplicate cultures were harvested, fixed and permeabilized, and labeled with an anti-human active caspase-3 antibody and PE-conjugated anti-rabbit IgG as described in Materials and methods. The percentage of GFP+(Env-expressing) and GFP-(Env-negative) cells that stained positive for active caspase-3 expression was determined by flow cytometry. The data shown are means of duplicate wells, and the error bars represent standard deviations.

suggests that increased pathogenicity by L-R5 HIV-1 strains probably does not involve cytotoxicity of cells surrounding those expressing Env. Previous studies have shown that Env-mediated bystander apoptosis may occur in cellular systems using transfection to over-express Env or with cell lines constitutively overexpressing Env (Algeciras-Schimnich et al., 2002; Garg and Blumenthal, 2006; Garg et al., 2007), infection with cell-free virions (Holm et al., 2004; Jacotot et al., 1997; Jekle et al., 2003), treatment with non-replicating virions (Holm et al., 2004), or soluble gp120 (Trushin et al., 2007), suggesting that death of bystander cells may occur when the cell exterior is exposed to Env or when Env is overexpressed on the cell surface. Our results suggest that bystander apoptosis is not likely to occur by Env expressed within single HIV-1 infected cells when expressed from the native HIV-1 RNA. However, this conclusion does not exclude the possibility that bystander cells may be destroyed when incorporated into syncytia or through hemifusion events when Env is expressed at higher levels or when cells are cultured at higher density. As hemifusion events are difficult to detect by flow cytometry, further studies using immunofluorescence are required to determine whether bystander apoptosis may occur in cells undergoing hemifusion with cells transduced with Env-expressing recombinant viruses. Indeed, hemifusion has been well described in cell-cell coculture systems using effector cells overexpressing Env on the cell surface and CD4+ T-cell targets [reviewed in (Garg and Blumenthal, 2008; Jacobs et al., 2008; Perfettini et al., 2005)]. Consistent with our results which highlight the dependence of Env-mediated fusion for apoptosis of Env-expressing cells, these studies showed that Env-mediated apoptosis of bystander cells through hemifusion was induced by gp41 (Garg and Blumenthal, 2006; Garg et al., 2007), and is influenced by the fusogenic properties of Env (Garg et al., 2009).

In conclusion, L-R5 Envs from two subjects with progressive R5 HIV-1 infection induced greater levels of cellular apoptosis than E-R5 Envs isolated from the same subjects during asymptomatic stages of HIV-1 infection, but only in Env-expressing cells, and only in cells where HIV-1 receptor levels were limiting and comparable to primary CD4+ T-cells. Further studies are required to confirm these results in the setting of viral replication. Our results showed that Env-mediated apoptosis depends on the fusion activity of Env. Increased apoptotic death of HIV-1 infected cells may contribute to CD4+ T-cell loss in subjects who experience progressive infection whilst harboring R5 HIV-1 variants.

## Materials and methods

### *Virus isolates*

In this study, we utilized a longitudinal panel of R5 HIV-1 viruses isolated sequentially from two subjects from asymptomatic ($n=2$

isolates) to late stages of HIV-1 infection ($n=2$ isolates). For the purpose of this study, the viruses isolated from patients with late stage HIV-1 infection are referred to as L-R5 viruses, and those isolated from the earlier times are referred to as E-R5 viruses. Viruses were isolated from sequential PBMC samples from subjects IK1 and IK2 and are designated IK1-E, IK1-L, IK2-E and IK2-L, respectively. The virus isolates were referred to previously as 435–531, 435–3415, 1047–314, and 1047–7418, respectively (Karlsson et al., 2004). The E-R5 isolates were obtained when both subjects had CD4+ T-cell counts of >600 cells/μl (50 and 41 months after infection for subjects IK1 and IK2, respectively), and the L-R5 isolates were obtained when both subjects were experiencing sustained immunological failure with CD4+ T-cell counts <300 cells/μl (95 and 130 months after infection for subjects IK1 and IK2, respectively) (Karlsson et al., 2004). A detailed characterization of the HIV-1 isolates including analysis of quasispecies diversity, coreceptor usage, replication kinetics, and clinical characteristics of the subjects from whom they were isolated, has been described previously (Jansson et al., 1999; Karlsson et al., 1994b, 2003, 2004).

### Cells

JC53 cells are derived from the HeLa cell line and stably express high levels of CD4, CXCR4 and CCR5 on the cell surface (Platt et al., 1998), and were cultured in DMEM supplemented with 10% (vol/vol) fetal calf serum (FCS), and 100 μg of penicillin and streptomycin per ml. Cf2-CD4/CCR5 cells (Yang et al., 2004) were cultured in DMEM supplemented with 10% (vol/vol) FCS, 100 μg of penicillin and streptomycin per ml, 0.5 mg of G418 per ml, and 0.1 mg of hygromycin per ml. Cf2-CD4/CXCR4 cells were constructed by transduction of the Cf2-CD4 cell line (Xiang et al., 2005) with pBABE-puro vectors expressing CXCR4 (Deng et al., 1997; Morgenstern and Land, 1990) followed by selection and expansion in DMEM supplemented with 10% (vol/vol) FCS, 100 μg of penicillin and streptomycin per ml, 0.5 mg of G418 per ml, and 1 μg of puromycin per ml. PM1 cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FCS, and 100 μg of penicillin and streptomycin per ml. 293T cells were cultured in DMEM supplemented with 10% (vol/vol) FCS, and 100 μg of penicillin and streptomycin per ml.

### PCR amplification, HIV-1 Env cloning, identification of functional Envs, and sequence analysis

The cloning of HIV-1 Envs derived from viruses IK1-E and IK1-L into the pSVIII-Env expression plasmid and their functional characterization including expression levels, gp120 processing and coreceptor usage has been described in detail previously (Sterjovski et al., 2007). Functional Envs were similarly cloned from viruses IK2-E and IK2-L. Briefly, viral RNA was isolated using a QIAmp UltraSense viral RNA isolation kit (Qiagen, Venlo, The Netherlands), according to the manufacturers' instructions. cDNA was reversed transcribed from viral RNA using SuperscriptIII RT (Invitrogen, Carlsbad, CA) and random hexamers, according to the manufacturers' protocol. An approximately 2.1 kb fragment spanning the *Kpn*I to *Bam*HI restriction sites in HIV-1 *env* (corresponding to nucleotides 6348 to 8478 in HXB2) was amplified by PCR using nested primers and Expand high fidelity DNA polymerase (Roche diagnostics, Basle, Switzerland). The outer primers were env1A and env1 M (Gao et al., 1996), and the inner primers were Env-*Kpn*I and Env-*Bam*HI (Gray et al., 2006; Ohagen et al., 2003). PCR cycling consisted of an initial denaturation step at 94 °C for 2 min followed by 9 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 2 min, then a further 20 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 2 min but with a 5 s increasing extension time for each cycle, followed by a final extension at 72 °C for 7 min. The products of 3 independent PCR reactions were purified and pooled, then cloned into the pSVIII-HXB2 Env expression plasmid by replacement of the 2.1 kb *Kpn*I to *Bam*HI HXB2 *env* fragment. Thus, the resulting Env clones contain the entire gp160 coding region of primary virus-derived *env* genes except for 36 amino acids at the N terminus and 105 amino acids at the C terminus, which are derived from HXB2. Functional Env clones were identified by the ability to support entry when pseudotyped onto Env-deficient GFP reporter viruses and used in single round entry assays in JC53 or Cf2-CD4/CCR5/CXCR4 cells, and by Western blot analysis of gp120/gp160 in transfected 293T cells and fusion assays. The coreceptor usage of functional Env clones was verified by single round entry assays in Cf2-CD4/CCR5 and Cf2-CD4/CXCR4 cells infected with Env-pseudotyped GFP reporter viruses, as described previously (Gray et al., 2006; Gray et al., 2009; He et al., 1997; Sterjovski et al., 2007). Envs were sequenced by Big Dye terminator sequencing (Applied Biosystems, Carlsbad, CA) and analyzed using a model 3100 Genetic Analyzer (Applied Biosystems).

### Viral vectors

The construction and characterization of the replication-defective psrHIVenvGFP vector, which expresses HIV-1 Env from a LTR promoter together with GFP from an IRES element, has been described in detail previously (LaBonte et al., 2000). In addition to Env and GFP, this vector expresses the HIV-1 Tat, Rev and Vif proteins and a C-terminally truncated, functionally defective Vpr protein derived from the HXBc2 strain of HIV-1. psrHIVenvGFP vectors containing the 2.1 kb *Kpn*I to *Bam*HI fragments of HXB2 or 89.6 Envs, or fusion-defective F/Y mutants of HXB2, 89.6 or ADA Envs in which phenylalanine at amino acid position 522 in each of these Envs was changed to tyrosine by PCR site directed mutagenesis have been described previously (LaBonte, Madani, and Sodroski, 2003; LaBonte et al., 2000) and were kindly provided by Dr Joseph Sodroski, Dana-Farber Cancer Institute, Boston. psrHIVenvGFP vectors containing Envs derived from the R5 ADA, JRCSF or YU2 strains of HIV-1, or one of eight different functional Envs derived from the primary R5 viruses IK1-E, IK1-L, IK2-E or IK2-L (clones IK1-E-1 and -2, IK1-L-1 and -2, IK2-E-1 and -2, and IK2-L-1 and -2, respectively) were constructed by replacing the 2.1 kb *Kpn*I to *Bam*HI fragment of psrHIVenvGFP with the corresponding fragments of pSVIII-Env plasmids encoding these Envs. The psrHIVΔenvGFP plasmid (LaBonte et al., 2000), which contains a deletion mutation in the *env* gene with an intact Rev-responsive element, and is otherwise isogenic to psrHIVenvGFP, was used as a negative control vector in the subsequent transduction studies to control for the effects of non-Env vector elements.

### Production of recombinant viruses

Replication-defective, Env-expressing transducing viruses pseudotyped with the vesicular stomatitis virus (VSV) G protein were produced by cotransfecting 293T cells with psrHIVenvGFP plasmid together with pCMVΔP1ΔenvpA, pCMV-G and pCMV-Rev (which all lack HIV-1 packaging signals and supply the HIV-1 *gag*, and *pol* gene products, the VSV G protein, and the HIV-1 Rev protein under the control of the cytomegalovirus immediate-early promoter, respectively, in *trans*), at a ratio of 10:10:2:1, as described previously (LaBonte et al., 2000). Transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. At 48 h post-transfection conditioned medium containing recombinant viruses was harvested, filtered through 0.45 μm filters, and stored in 1 ml aliquots at −80 °C.

### Titration of recombinant viruses

The recombinant viruses were titrated on JC53 or PM1 cells, to determine an appropriate dilution to achieve approximately 5–10% transduction efficiency in each cell type. Sub-confluent monolayers of

JC53 cells cultured in 24-well plates, or PM1 cells cultured at a density of $5\times10^5$ cells/ml in 24-well plates, were transduced for 16 h with serial dilutions of each recombinant virus whilst maintaining a constant total volume of culture medium, after which the medium was changed and the cells were incubated for a further 48 h at 37 °C. The cells were then collected, washed in phosphate-buffered saline, fixed in 4% (wt/vol) paraformaldehyde and analyzed for GFP expression using a Becton Dickinson FACSCalibur flow cytometer.

*Transduction of cells and active caspase-3 staining*

JC53 or PM1 cells were cultured in duplicate wells of 24-well plate as described above, and transduced with a sufficient titer of recombinant virus to achieve infection of approximately 5–10% of cells. These culture conditions were found to be optimal for ensuring that the majority of the cells transduced with Env and GFP remain as single cells and not syncytia (LaBonte et al., 2000). After 16 h the culture medium was changed, and cells from duplicate transductions were harvested 24, 48, 72 and 96 h later for analysis of active caspase-3. At these timepoints, cells were washed in PBS and then fixed and permeabilized with 100 µl cytofix-cytoperm solution (Pharmingen, San Diego, CA.) for 30 min at 4°. After washing twice with perm/wash buffer (Pharmingen), the cells were blocked with 1% (wt/vol) bovine serum albumin (BSA) followed by incubation with a 1:250 dilution of anti-human active caspase-3 antibody (Promega, Madison, WI) for 1 h at room temperature. Cells were washed twice with perm/wash buffer (Pharmingen) and then incubated with PE-conjugated F' (ab) anti-rabbit IgG antibody (Rockland Immunochemicals) for 45 min at room temperature. Cells were then washed twice with PBS, resuspended in 200 µl PBS and analyzed for GFP and active caspase-3 expression by flow cytometry.

*Immunofluorescence*

JC53 cells were cultured on chamber slides (ibidi, Munich, Germany) and transduced with recombinant virus expressing HXB2, 89.6 or ADA Env. Cells transduced with recombinant virus expressing the Env mutant (ΔEnv) were included as a negative control for detection of Env expression. At 48 h post-transduction, the slides were washed in PBS, fixed and permeabilized in PBS containing 4% (wt/vol) paraformaldehyde and 0.1% (vol/vol) Triton-X100 for 30 min at room temperature, and blocked in PBS containing 1% (wt/vol) BSA and 5% (vol/vol) normal goat serum (Chemicon, Temecula, CA), for 90 min at room temperature. Cells were then incubated with a 1:200 dilution of BB10 antisera in PBS containing 1% (wt/vol) BSA and 0.5% (vol/vol) Tween-20 for 1 h at room temperature. BB10 is reactive against HIV-1 Env and Gag proteins but is not reactive against HIV-1 regulatory/accessory proteins (Gorry et al., 1998, 1999). Thus, in the transductions BB10 antisera is specific for HIV-1 Env. Cells transduced with recombinant virus expressing the ΔEnv mutant were included to confirm the specificity of BB10 for Env in these experiments. Cells were then washed 5 times in PBS containing 0.5% (vol/vol) Tween-20, and then incubated with a 1:500 dilution of Texas Red-conjugated anti-human antibody in PBS containing 1% (wt/vol) BSA and 0.5% (vol/vol) Tween-20 for 45 min at room temperature. Cells were washed in PBS and images were captured in a z series on a charge-coupled device camera (CoolSnap HQ, Photometrics) through a $60\times1.42$ numerical aperture oil immersion lens on a DeltaVision microscope (Applied Precision, Issaquah, WA), and deconvolved using SoftWoRx deconvolution software (Applied Precision).

*Env Western blotting*

For analysis of Env expression, JC53 cells transduced with recombinant viruses expressing HXB2, 89.6 or ADA Env, or with recombinant virus expressing the ΔEnv mutant, were harvested at 48 h post-transduction, washed in PBS, resuspended in ice-cold lysis buffer [10 mM Tris (pH 8.0), 0.5% (vol/vol) Triton X100, 150 mM NaCl] containing a cocktail of protease inhibitors for 20 min, followed by centrifugation at 16,000×*g* for 10 min to pellet cellular debris. Cell lysates were separated in 8.5% SDS-PAGE gels and analyzed by Western blotting using a 1:8000 dilution of rabbit anti-gp120 polyclonal antisera as described previously (Gray et al., 2006; Sterjovski et al., 2007). Env proteins were visualized using a 1:16,000 dilution of horseradish peroxidase-conjugated anti rabbit IgG antibody (Amersham Scientific, Piscataway, NJ) and SuperSignal enhanced chemiluminescence (Thermo Fisher, Rockford, IL).

*Nucleotide sequence accession numbers*

The new Env nucleotide sequences reported here have been assigned GenBank accession numbers GU074012 to GU074015.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.10.029.

## References

Algeciras-Schimnich, A., Vlahakis, S.R., Villasis-Keever, A., Gomez, T., Heppelmann, C.J., Bou, G., Paya, C.V., 2002. CCR5 mediates Fas- and caspase-8 dependent apoptosis of both uninfected and HIV infected primary human CD4T cells. Aids 16, 1467–1478.

Bjorndal, A., Deng, H., Jansson, M., Fiore, J.R., Colognesi, C., Karlsson, A., Albert, J., Scarlatti, G., Littman, D.R., Fenyo, E.M., 1997. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. J. Virol. 71, 7478–7487.

Borggren, M., Repits, J., Kuylenstierna, C., Sterjovski, J., Churchill, M.J., Purcell, D.F., Karlsson, A., Albert, J., Gorry, P.R., Jansson, M., 2008. Evolution of DC-SIGN use revealed by fitness studies of R5 HIV-1 variants emerging during AIDS progression. Retrovirology 5, 28.

Briggs, D.R., Tuttle, D.L., Sleasman, J.W., Goodenow, M.M., 2000. Envelope V3 amino acid sequence predicts HIV-1 phenotype (co-receptor usage and tropism for macrophages). Aids 14, 2937–2939.

Cayabyab, M., Karlsson, G.B., Etemad-Moghadam, B.A., Hofmann, W., Steenbeke, T., Halloran, M., Fanton, J.W., Axthelm, M.K., Letvin, N.L., Sodroski, J.G., 1999. Changes in human immunodeficiency virus type 1 envelope glycoproteins responsible for the pathogenicity of a multiply passaged simian-human immunodeficiency virus (SHIV-HXBc2). J. Virol. 73, 976–984.

Connor, R.I., Sheridan, K.E., Ceradini, D., Choe, S., Landau, N.R., 1997. Change in coreceptor use coreceptor use correlates with disease progression in HIV-1-infected individuals. J. Exp. Med. 185, 621–628.

de Roda Husman, A.M., Schuitemaker, H., 1998. Chemokine receptors and the clinical course of HIV-1 infection. Trends Microbiol. 6, 244–249.

de Roda Husman, A.M., van Rij, R.P., Blaak, H., Broersen, S., Schuitemaker, H., 1999. Adaptation to promiscuous usage of chemokine receptors is not a prerequisite for human immunodeficiency virus type 1 disease progression. J. Infect. Dis. 180, 1106–1115.

Deng, H.K., Unutmaz, D., KewalRamani, V.N., Littman, D.R., 1997. Expression cloning of new receptors used by simian and human immunodeficiency viruses. Nature 388, 296–300.

Etemad-Moghadam, B., Sun, Y., Nicholson, E.K., Fernandes, M., Liou, K., Gomila, R., Lee, J., Sodroski, J., 2000. Envelope glycoprotein determinants of increased fusogenicity in a pathogenic simian-human immunodeficiency virus (SHIV-KB9) passaged in vivo. J. Virol. 74, 4433–4440.

Etemad-Moghadam, B., Rhone, D., Steenbeke, T., Sun, Y., Manola, J., Gelman, R., Fanton, J.W., Racz, P., Tenner-Racz, K., Axthelm, M.K., Letvin, N.L., Sodroski, J., 2001. Membrane-fusing capacity of the human immunodeficiency virus envelope

proteins determines the efficiency of CD+ T-cell depletion in macaques infected by a simian-human immunodeficiency virus. J. Virol. 75, 5646–5655.
Fais, S., Lapenta, C., Santini, S.M., Spada, M., Parlato, S., Logozzi, M., Rizza, P., Belardelli, F., 1999. Human immunodeficiency virus type 1 strains R5 and X4 induce different pathogenic effects in hu-PBL-SCID mice, depending on the state of activation/differentiation of human target cells at the time of primary infection. J. Virol. 73, 6453–6459.
Gao, F., Morrison, S.G., Robertson, D.L., Thornton, C.L., Craig, S., Karlsson, G., Sodroski, J., Morgado, M., Galvao-Castro, B., von Briesen, H., et al., 1996. Molecular cloning and analysis of functional envelope genes from human immunodeficiency virus type 1 sequence subtypes A through G. The WHO and NIAID Networks for HIV Isolation and Characterization. J. Virol. 70, 1651–1667.
Garg, H., Blumenthal, R., 2006. HIV gp41-induced apoptosis is mediated by caspase-3-dependent mitochondrial depolarization, which is inhibited by HIV protease inhibitor nelfinavir. J. Leukoc. Biol. 79, 351–362.
Garg, H., Blumenthal, R., 2008. Role of HIV Gp41 mediated fusion/hemifusion in bystander apoptosis. Cell Mol. Life Sci. 65, 3134–3144.
Garg, H., Joshi, A., Freed, E.O., Blumenthal, R., 2007. Site-specific mutations in HIV-1 gp41 reveal a correlation between HIV-1-mediated bystander apoptosis and fusion/hemifusion. J. Biol. Chem. 282, 16899–16906.
Garg, H., Joshi, A., Blumenthal, R., 2009. Altered bystander apoptosis induction and pathogenesis of enfuvirtide-resistant HIV type 1 Env mutants. AIDS Res. Hum. Retroviruses 25, 811–817.
Glushakova, S., Baibakov, B., Margolis, L.B., Zimmerberg, J., 1995. Infection of human tonsil histocultures: a model for HIV pathogenesis. Nature Med. 1, 1320–1322.
Glushakova, S., Grivel, J.C., Fitzgerald, W., Sylwester, A., Zimmerberg, J., Margolis, L.B., 1998. Evidence for the HIV-1 phenotype switch as a causal factor in acquired immunodeficiency. Nature Med. 4, 346–349.
Gorry, P., Purcell, D., Howard, J., McPhee, D., 1998. Restricted HIV-1 infection of human astrocytes: potential role of nef in the regulation of virus replication. J. Neurovirol. 4, 377–386.
Gorry, P.R., Sterjovski, J., Churchill, M., Witlox, K., Gray, L., Cunningham, A., Wesselingh, S., 2004. The role of viral coreceptors and macrophage tropism in human immunodeficiency virus type 1 disease progression. Sex Health 1, 23–34.
Gorry, P.R., Howard, J.L., Churchill, M.J., Anderson, J.L., Cunningham, A., Adrian, D., McPhee, D.A., Purcell, D.F., 1999. Diminished production of human immunodeficiency virus type 1 in astrocytes results from inefficient translation of gag, env, and nef mRNAs despite efficient expression of Tat and Rev. J. Virol. 73, 352–361.
Gray, L., Sterjovski, J., Churchill, M., Ellery, P., Nasr, N., Lewin, S.R., Crowe, S.M., Wesselingh, S., Cunningham, A.L., Gorry, P.R., 2005. Uncoupling coreceptor usage of human immunodeficiency virus type 1 (HIV-1) from macrophage tropism reveals biological properties of CCR5-restricted HIV-1 isolates from patients with acquired immunodeficiency syndrome. Virology 337, 384–398.
Gray, L., Churchill, M.J., Keane, N., Sterjovski, J., Ellett, A.M., Purcell, D.F.J., Poumbourios, P., Kol, C., Wang, B., Saksena, N., Wesselingh, S.L., Price, P., French, M., Gabuzda, D., Gorry, P.R., 2006. Genetic and functional analysis of R5X4 human immunodeficiency virus type 1 envelope glycoprotiens derived from two individuals homozygous for the CCR5delta32 allele. J. Virol. 80, 3684–3691.
Gray, L., Roche, M., Churchill, M.J., Sterjovski, J., Ellett, A., Poumbourios, P., Sheffief, S., Wang, B., Saksena, N., Purcell, D.F., Wesselingh, S., Cunningham, A.L., Brew, B.J., Gabuzda, D., Gorry, P.R., 2009. Tissue-specific sequence alterations in the human immunodeficiency virus type 1 envelope favoring CCR5 usage contribute to persistence of dual-tropic virus in the brain. J. Virol. 83, 5430–5441.
Grivel, J.C., Margolis, L.B., 1999. CCR5- and CXCR4-tropic HIV-1 are equally cytopathic for their T-cell targets in human lymphoid tissue. Nature Med. 5, 344–346.
Harouse, J.M., Gettie, A., Tan, R.C., Blanchard, J., Cheng-Mayer, C., 1999. Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. Science 284, 816–819.
He, J., Chen, Y., Farzan, M., Choe, H., Ohagen, A., Gartner, S., Busciglio, J., Yang, X., Hofmann, W., Newman, W., Mackay, C.R., Sodroski, J., Gabuzda, D., 1997. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature 385, 645–649.
Holm, G.H., Zhang, C., Gorry, P.R., Peden, K.W., Schols, D., De Clercq, E., Gabuzda, D., 2004. Apoptosis of bystander T cells induced by human immunodeficiency virus type 1 with increased envelope/receptor affinity and coreceptor binding site exposure. J. Virol. 78, 4541–4551.
Jacobs, A., Garg, H., Viard, M., Raviv, Y., Puri, A., Blumenthal, R., 2008. HIV-1 envelope glycoprotein-mediated fusion and pathogenesis: implications for therapy and vaccine development. Vaccine 26, 3026–3035.
Jacotot, E., Krust, B., Callebaut, C., Laurent-Crawford, A.G., Blanco, J., Hovanessian, A.G., 1997. HIV-1 envelope glycoproteins-mediated apoptosis is regulated by CD4 dependent and independent mechanisms. Apoptosis 2, 47–60.
Jansson, M., Popovic, M., Karlsson, A., Cocchi, F., Rossi, P., Albert, J., Wigzell, H., 1996. Sensitivity to inhibition by beta-chemokines correlates with biological phenotypes of primary HIV-1 isolates. Proc. Natl. Acad. Sci. U.S.A. 93, 15382–15387.
Jansson, M., Backstrom, E., Bjorndal, A., Holmberg, V., Rossi, P., Fenyo, E.M., Popovic, M., Albert, J., Wigzell, H., 1999. Coreceptor usage and RANTES sensitivity of non-syncytium-inducing HIV-1 isolates obtained from patients with AIDS. J. Hum. Virol. 2, 325–338.
Jekle, A., Keppler, O.T., De Clercq, E., Schols, D., Weinstein, M., Goldsmith, M.A., 2003. In vivo evolution of human immunodeficiency virus type 1 toward increased pathogenicity through CXCR4-mediated killing of uninfected CD4 T cells. J. Virol. 77, 5846–5854.
Karlsson, A., Parsmyr, K., Aperia, K., Sandstrom, E., Fenyo, E.M., Albert, J., 1994a. MT-2 cell tropism of human immunodeficiency virus type 1 isolates as a marker for response to treatment and development of drug resistance. J. Infect. Dis. 170, 1367–1375.
Karlsson, A., Parsmyr, K., Sandstrom, E., Fenyo, E.M., Albert, J., 1994b. MT-2 cell tropism as prognostic marker for disease progression in human immunodeficiency virus type 1 infection. J. Clin. Microbiol. 32, 364–370.
Karlsson, G.B., Halloran, M., Li, J., Park, I.W., Gomila, R., Reimann, K.A., Axthelm, M.K., Iliff, S.A., Letvin, N.L., Sodroski, J., 1997. Characterization of molecularly cloned simian-human immunodeficiency viruses causing rapid CD4+ lymphocyte depletion in rhesus monkeys. J. Virol. 71, 4218–4225.
Karlsson, G.B., Halloran, M., Schenten, D., Lee, J., Racz, P., Tenner-Racz, K., Manola, J., Gelman, R., Etemad-Moghadam, B., Desjardins, E., Wyatt, R., Gerard, N.P., Marcon, L., Margolin, D., Fanton, J., Axthelm, M.K., Letvin, N.L., Sodroski, J., 1998. The envelope glycoprotein ectodomains determine the efficiency of CD4+ T lymphocyte depletion in simian-human immunodeficiency virus-infected macaques. J. Exp. Med. 188, 1159–1171.
Karlsson, I., Antonsson, L., Shi, Y., Karlsson, A., Albert, J., Leitner, T., Olde, B., Owman, C., Fenyo, E.M., 2003. HIV biological variability unveiled: frequent isolations and chimeric receptors reveal unprecedented variation of coreceptor use. AIDS 17, 2561–2569.
Karlsson, I., Antonsson, L., Shi, Y., Oberg, M., Karlsson, A., Albert, J., Olde, B., Owman, C., Jansson, M., Fenyo, E.M., 2004. Coevolution of RANTES sensitivity and mode of CCR5 receptor use by human immunodeficiency virus type 1 of the R5 phenotype. J. Virol. 78, 11807–11815.
Koning, F.A., Kwa, D., Boeser-Nunnink, B., Dekker, J., Vingerhoed, J., Hiemstra, H., Schuitemaker, H., 2003. Decreasing sensitivity to RANTES (regulated on activation, normally T cell-expressed and -secreted) neutralization of CC chemokine receptor 5-using, non-syncytium-inducing virus variants in the course of human immunodeficiency virus type 1 infection. J. Infect. Dis. 188, 864–872.
Koot, M., Keet, I.P., Vos, A.H., de Goede, R.E., Roos, M.T., Coutinho, R.A., Miedema, F., Schellekens, P.T., Tersmette, M., 1993. Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. Ann. Intern. Med. 118, 681–688.
LaBonte, J.A., Patel, T., Hofmann, W., Sodroski, J., 2000. Importance of membrane fusion mediated by human immunodeficiency virus envelope glycoproteins for lysis of primary CD4-positive T cells. J. Virol. 74, 10690–10698.
LaBonte, J.A., Madani, N., Sodroski, J., 2003. Cytolysis by CCR5-using human immunodeficiency virus type 1 envelope glycoproteins is dependent on membrane fusion and can be inhibited by high levels of CD4 expression. J. Virol. 77, 6645–6659.
Li, S., Juarez, J., Alali, M., Dwyer, D., Collman, R., Cunningham, A., Naif, H.M., 1999. Persistent CCR5 utilization and enhanced macrophage tropism by primary blood human immunodeficiency virus type 1 isolates from advanced stages of disease and comparison to tissue-derived isolates. J. Virol. 73, 9741–9755.
Liu, Z.Q., Muhkerjee, S., Sahni, M., McCormick-Davis, C., Leung, K., Li, Z., Gattone 2nd, V.H., Tian, C., Doms, R.W., Hoffman, T.L., Raghavan, R., Narayan, O., Stephens, E.B., 1999. Derivation and biological characterization of a molecular clone of SHIV (KU-2) that causes AIDS, neurological disease, and renal disease in rhesus macaques. Virology 260, 295–307.
Milich, L., Margolin, B., Swanstrom, R., 1993. V3 loop of the human immunodeficiency virus type 1 Env protein: interpreting sequence variability. J. Virol. 67, 5623–5634.
Morgenstern, J.P., Land, H., 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. 18, 3587–3596.
Ohagen, A., Devitt, A., Kunstman, K.J., Gorry, P.R., Rose, P.P., Korber, B., Taylor, J., Levy, R., Murphy, R.L., Wolinsky, S.M., Gabuzda, D., 2003. Genetic and functional analysis of full-length human immunodeficiency virus type 1 env genes derived from brain and blood of patients with AIDS. J. Virol. 77, 12336–12345.
Perfettini, J.L., Castedo, M., Roumier, T., Andreau, K., Nardacci, R., Piacentini, M., Kroemer, G., 2005. Mechanisms of apoptosis induction by the HIV-1 envelope. Cell Death Differ. 12 (Suppl. 1), 916–923.
Picchio, G.R., Gulizia, R.J., Wehrly, K., Chesebro, B., Mosier, D.E., 1998. The cell tropism of human immunodeficiency virus type 1 determines the kinetics of plasma viremia in SCID mice reconstituted with human peripheral blood leukocytes. J. Virol. 72, 2002–2009.
Platt, E.J., Wehrly, K., Kuhmann, S.E., Chesebro, B., Kabat, D., 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. J. Virol. 72, 2855–2864.
Repits, J., Oberg, M., Esbjornsson, J., Medstrand, P., Karlsson, A., Albert, J., Fenyo, E.M., Jansson, M., 2005. Selection of human immunodeficiency virus type 1 R5 variants with augmented replicative capacity and reduced sensitivity to entry inhibitors during severe immunodeficiency. J. Gen. Virol. 86, 2859–2869.
Repits, J., Sterjovski, J., Badia-Martinez, D., Mild, M., Gray, L., Churchill, M.J., Purcell, D.F., Karlsson, A., Albert, J., Fenyo, E.M., Achour, A., Gorry, P.R., Jansson, M., 2008. Primary HIV-1 R5 isolates from end-stage disease display enhanced viral fitness in parallel with increased gp120 net charge. Virology 379, 125–134.
Si, Z., Gorry, P., Babcock, G., Owens, C.M., Cayabyab, M., Phan, N., Sodroski, J., 2004. Envelope glycoprotein determinants of increased entry in a pathogenic simian-human immunodeficiency virus (SHIV-HXBc2P 3.2) passaged in monkeys. AIDS Res. Hum. Retroviruses 20, 163–173.
Sodroski, J., Goh, W.C., Rosen, C., Campbell, K., Haseltine, W.A., 1986. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. Nature 322, 470–474.
Stephens, E.B., Joag, S.V., Sheffer, D., Liu, Z.Q., Zhao, L., Mukherjee, S., Foresman, L., Adany, I., Li, Z., Pinson, D., Narayan, O., 1996. Initial characterization of viral sequences from a SHIV-inoculated pig-tailed macaque that developed AIDS. J. Med. Primatol. 25, 175–185.
Stephens, E.B., Mukherjee, S., Sahni, M., Zhuge, W., Raghavan, R., Singh, D.K., Leung, K., Atkinson, B., Li, Z., Joag, S.V., Liu, Z.Q., Narayan, O., 1997. A cell-free stock of simian-

human immunodeficiency virus that causes AIDS in pig-tailed macaques has a limited number of amino acid substitutions in both SIVmac and HIV-1 regions of the genome and has offered cytotropism. Virology 231, 313–321.
Sterjovski, J., Churchill, M.J., Wesselingh, S.L., Gorry, P.R., 2006. HIV-1 entry inhibitors: classes, applications and factors affecting potency. Curr. HIV Res. 4, 387–400.
Sterjovski, J., Churchill, M.J., Ellett, A., Gray, L.R., Roche, M.J., Dunfee, R.L., Purcell, D.F., Saksena, N., Wang, B., Sonza, S., Wesselingh, S.L., Karlsson, I., Fenyo, E.M., Gabuzda, D., Cunningham, A.L., Gorry, P.R., 2007. Asn 362 in gp120 contributes to enhanced fusogenicity by CCR5-restricted HIV-1 envelope glycoprotein variants from patients with AIDS. Retrovirology 4, 89.
Tersmette, M., Gruters, R.A., de Wolf, F., de Goede, R.E., Lange, J.M., Schellekens, P.T., Goudsmit, J., Huisman, H.G., Miedema, F., 1989. Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates. J. Virol. 63, 2118–2125.
Trushin, S.A., Algeciras-Schimnich, A., Vlahakis, S.R., Bren, G.D., Warren, S., Schnepple, D.J., Badley, A.D., 2007. Glycoprotein 120 binding to CXCR4 causes p38-dependent primary T cell death that is facilitated by, but does not require cell-associated CD4. J. Immunol. 178, 4846–4853.
Tuttle, D.L., Anders, C.B., Aquino-De Jesus, M.J., Poole, P.P., Lamers, S.L., Briggs, D.R., Pomeroy, S.M., Alexander, L., Peden, K.W., Andiman, W.A., Sleasman, J.W., Goodenow, M.M., 2002. Increased replication of non-syncytium-inducing HIV type 1 isolates in monocyte-derived macrophages is linked to advanced disease in infected children. AIDS Res. Hum. Retroviruses 18, 353–362.
Xiang, S.H., Farzan, M., Si, Z., Madani, N., Wang, L., Rosenberg, E., Robinson, J., Sodroski, J., 2005. Functional mimicry of a human immunodeficiency virus type 1 coreceptor by a neutralizing monoclonal antibody. J. Virol. 79, 6068–6077.
Yang, X., Tomov, V., Kurteva, S., Wang, L., Ren, X., Gorny, M.K., Zolla-Pazner, S., Sodroski, J., 2004. Characterization of the outer domain of the gp120 glycoprotein from human immunodeficiency virus type 1. J. Virol. 78, 12975–12986.