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Apoptosis induced in synchronized human immunodeficiency virus type 1-infected primary peripheral blood mononuclear cells is detected after the peak of CD4+ T-lymphocyte loss and is dependent on the tropism of the gp120 envelope glycoprotein

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Abstract

Disease progression in human immunodeficiency virus type-1 (HIV-1)-infected individuals is frequently accompanied by declining CD4 cell numbers and the acquisition of a T-tropic (X4) or dual tropic (R5X4) phenotype. Understanding the mechanism of CD4 cell loss in HIV-1 infection is essential for the development of effective therapeutic strategies. In this study, donor populations of peripheral blood mononuclear cells (PBMCs) were selected for their ability to support an equivalent acute infection by both R5 and X4 virus phenotypes. This demonstrated that CD4+ T-lymphocyte loss was due to the gp120 region of Env and was replication independent. Furthermore, apoptosis was only detected in cells infected with an X4 virus after the majority of CD4+ T-lymphocyte loss had occurred. These observations indicate that the CD4+ T-lymphocyte loss in an X4 HIV-1 infection is not directly mediated by apoptosis, although apoptosis may be induced in the remaining cell population as a consequence of this CD4+ T-lymphocyte loss.

Keywords: HIV-1; Envelope; Tropism; Apoptosis; PBMC; Nef

Introduction

Disease progression in human immunodeficiency virus type 1 (HIV-1)-infected individuals is accompanied by a decline in CD4+ lymphocyte numbers. These cells play a major role in the regulation and function of the immune response, and their loss predisposes the host to life-threatening opportunistic infections. A possible mediator of this cell loss is virus-induced apoptosis. Previous studies have shown that peripheral blood mononuclear cells (PBMCs) isolated from HIV-1-infected individuals undergo spontaneous and activation-induced apoptosis more frequently than PBMCs from uninfected individuals (Gougeon, 1996; Groux et al., 1992; Karmochkine et al., 1998; Meyaard et al., 1992; Oyaizu et al., 1993, 1995; Pandolfi et al., 1995). However, the mechanism underlying HIV-1-induced apoptosis, and indeed whether apoptosis is truly the key proponent of CD4+ lymphocyte loss, remains poorly understood.

Apoptosis is a genetically controlled process that mediates the self-destruction of redundant, aberrant, or injured cells. Most animal viruses have developed mechanisms to delay apoptosis to allow the replication of viral progeny (Benedict et al., 2002). However, animal viruses also induce apoptosis later in the infection cycle to facilitate the spread

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of viral progeny and avoid activation of the host's immune response. Several HIV-1 proteins, including Tat, Vpr, Vpu, Nef, and Env, have been implicated in both pro- and anti-apoptotic processes (Akari et al., 2001; Casella et al., 1999; Roshal et al., 2001).

The accessory protein Nef, although not essential for viral replication, exerts a significant effect on replication efficiency and pathogenesis (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995; Salvi et al., 1998). Nef can downmodulate cell surface expression of CD4 (Garcia and Miller, 1991) and MHC class I (Schwartz et al., 1996) in HIV-1-infected cells and has more recently been shown to modulate T-cell signaling (Renkema and Saksela, 2000) through a TCR-associated signaling complex (Simmons et al., 2001). Stimulation of the T-cell receptor by Nef appears to prime the infected cell for HIV-1 replication. But, perhaps more significant is the ability of Nef to facilitate macrophage-mediated resistance to apoptosis by inducing an anti-apoptotic response in the early phase of virus replication (Mahlknecht et al., 2000) and promoting a pro-apoptotic response in the late phase of virus replication (Greenway et al., 2003).

The phenotype of the HIV-1 Env glycoprotein is an important determinant of virus tropism and pathogenesis. Env mediates the binding and entry of virus into cells expressing the CD4 receptor. However, virus infection also requires the interaction of Env with a secondary co-receptor. Viral infection of primary macrophages (M-tropic) requires the expression and use of the CCR5 co-receptor (R5), whereas virus infection of CD4+ T-lymphocytes and T-cell lines (T-tropic) requires expression and use of the CXCR4 co-receptor (X4). Viruses with an R5 phenotype are found early in infection, whereas X4 or dual-tropic viruses (R5X4) are more cytopathic and predominate late in the infection process. These differences suggest that changes in Env that accompany disease progression may be directly related to the pathogenicity of the virus.

In vitro, cross-linking of CD4 with a recombinant form of the gp120 Env subunit (Laurent-Crawford et al., 1993) or exogenous expression of HIV-1 Env (Laurent-Crawford et al., 1995) has been shown to promote apoptosis in T-cell lines. In HIV-1-infected individuals, the ability of PBMC to undergo apoptosis has been correlated with increased viral load and disease progression (Gougeon et al., 1996; Pandolfi et al., 1995). Therefore, HIV-1-mediated apoptosis may be induced by the increase in viral load that accompanies disease progression or by a change in viral phenotype from R5 to X4 or R5X4.

A difficulty associated with the study of HIV-1 Env phenotype in vitro is the innate differences in the replication ability and kinetics of these virus types in cell lines and primary cell cultures. This had led to the conclusion that differences in CD4+ lymphocyte depletion from PBMC infected with primary M- and T-tropic virus strains may be related to viral replication (Zhang et al., 1997). However, similar replication kinetics, achieved by infection of PBMC with different multiplicities of infection, of isogenic X4 and R5 virus phenotypes indicated that CD4+ lymphocyte loss was greater in X4-infected cultures (Penn et al., 1999). Thus suggesting that CD4+ lymphocyte loss was independent of virus replication kinetics. The mechanism of HIV-1-mediated cell loss also remains controversial following the observation that death of CD4+ lymphocytes was primarily induced by necrosis (Lenardo et al., 2002) despite previous studies reporting the detection of apoptosis in X4-infected PBMC (Gandhi et al., 1998; Herbein et al., 1998b) or a combination of both apoptosis and necrosis (Plymale et al., 1999).

In the current study, the effect of viral phenotype, with respect to Env, on CD4+ lymphocyte loss and induction of apoptosis was investigated in a model of acute virus replication in primary activated, synchronized PBMC. In a detailed kinetic analysis, it was found that cell loss occurred in PBMC infected with HIV-1 with an X4 but not R5 Env phenotype and that apoptosis was only detected in X4-infected cells after the loss of CD4+ T-lymphocytes. Our data suggest that apoptosis associated with HIV-1 infection is a consequence of CD4+ T-lymphocyte loss and not the primary mediator.

Results

CD4 downmodulation is followed by cell death in PBMC infected with T-tropic/X4 isolates of HIV-1

Primary HIV-1 and reference isolates were used to investigate the relationship between viral tropism and virulence in PBMC. MBC200 was isolated from PBMC and replicates in primary T-lymphocytes and T-cell lines and uses CXCR4 for viral entry (Kiernan et al., 1990; Oelrichs et al., 2000). MBC925 and MBC676 were isolated from CSF and brain, respectively, and replicate in macrophages and PBMC (Cameron et al., 1996; Oelrichs et al., 2000). Both viruses have been shown to use CCR5 for viral entry (Oelrichs et al., 2000). These isolates were used to infect mixed populations of PHA-stimulated PBMC (PHA-PBMC). Cell-free reverse transcriptase (RT) activity indicated that all viruses replicated to high levels in PBMC and peaked between days 7 and 11 postinfection (Fig. 1a). Flow cytometry analysis for CD4+/CD3+ PBMC indicated that CD4 expression had decreased by day 7 postinfection in all HIV-1-infected cultures relative to the mock-infected control, albeit more dramatically in cultures infected with LAI and MBC200 (Fig. 1b). By 14 days postinfection, there were no CD4+ cells detected in cultures infected with LAI and MBC200, whereas the percent of CD4+ cells in cultures infected with AD8 and MBC925 was reduced by approximately 50% relative to mock-infected cultures and by 75% in MBC676-infected cultures.

To determine whether the loss of CD4 expression was due to CD4 receptor downregulation (Chen et al., 1996) or

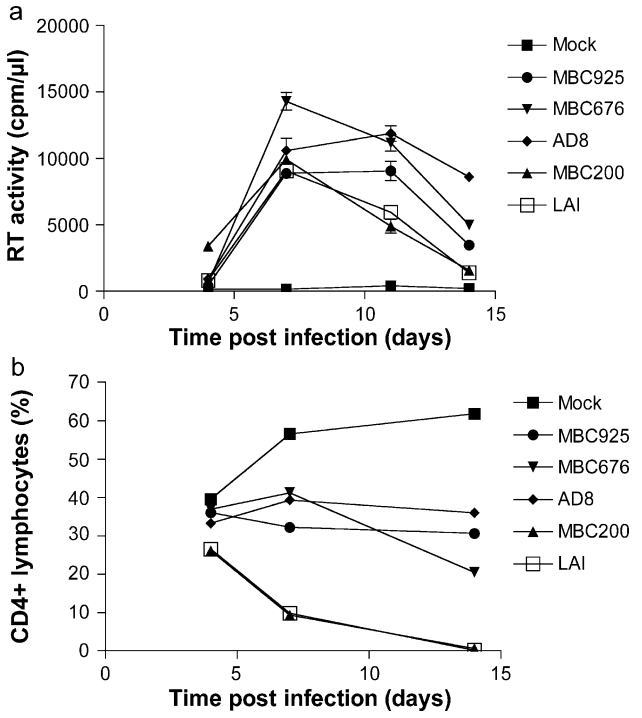

Fig. 1. Characterization of CD4 antigen expression on PBMC infected with primary HIV-1 isolates. Mixed populations of PHA-PBMC were infected with primary HIV-1 isolates MBC200, MBC925, and MBC676 or molecular clones LAI and AD8 at a moi of 0.0001 or mock infected. Virus replication kinetics were determined by cell-free RT activity (a). The percentage of CD4+ lymphocytes was determined by flow cytometry analysis of cells labeled with anti-CD4-FITC/anti-CD3-PE antibodies (b). Results of a representative infection are shown.

CD4+ lymphocyte death, we looked for an increase in the CD4 – /CD3+ lymphocyte population that could not be attributed to the outgrowth of a CD8+ lymphocyte population. This was achieved by comparing the percentage of CD4 – /CD3+ cells with the percentage of CD4 – /CD8+ cells obtained through parallel dual fluorescence labeling of infected cell cultures (Fig. 2). In LAI- and MBC200-infected cultures, CD4 downmodulation was followed by cell death 14 days postinfection. In contrast, the loss of CD4+ cells from cultures infected with M-tropic/R5 HIV-1 was mostly due to the downmodulation of CD4 expression. This population is represented as CD4 – /CD3+ cells that are not CD8+ (summarized Table 1). Our results demonstrate that both T-tropic/X4 and M-tropic/R5 HIV-1 phenotypes downmodulate CD4 expression following initial viral entry. However, only in PBMC populations infected with the T-tropic/X4 phenotype was this CD4 downmodulation followed by significant cell death.

Interestingly, unlike the other M-tropic isolates studied here, the decrease in CD4 expression observed in cultures infected with MBC676 was also partly due to cell loss despite the M-tropic/R5 phenotype of this viral isolate (Table 1). This may be attributable to strong virulence described for M-tropic HIV-1 isolated from the brain (Gorry et al., 2002a).

The Nef accessory protein does not mediate CD4+ lymphocyte loss in primary PBMC cultures

The HIV-1 accessory protein Nef has been implicated in CD4+ lymphocyte survival (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995; Mahlknecht et al., 2000; Salvi et al., 1998). HIV-1 isolates from the Sydney blood bank cohort (SBBC) encode a natural deletion within the *nef* gene (Deacon et al., 1995) and consequently do not express a Nef protein. The CD4+ lymphocyte survival of two SBBC isolates, MBCC18 (R5) and MBCD36 (dual tropic R5/X4), was used to investigate the importance of Nef in CD4+ lymphocyte survival. In addition, NL4.3 was included as a prototypic X4 virus, and a chimeric clone NLAD composed of the gp120 region of AD8 (R5) in an NL4.3 background was included as a prototypic R5 virus. The co-receptor use of these viruses was confirmed in Cf2-Luc cells (Table 2). Viruses derived from *nef*-deleted clones of NL4.3 (NL4.3Δnef) and NLAD (NLADΔnef) were included to directly assess the effect of Nef relative to Env phenotype on CD4+ lymphocyte survival.

The kinetics of replication of MBCD36 were similar to that of the molecular clones of NL4.3, NL4.3Δnef, NLAD, and NLADΔnef. Whereas the replication kinetics of the primary isolate MBCC18 were delayed by 2 days in the same donor cell population (Fig. 3a). Analysis of the percent of CD4+ lymphocytes in the total population indicated that minimal cell killing occurred in PBMC infected with a virus with an R5 phenotype (Fig. 3b; MBCC18, NLAD, or NLADΔnef). However, CD4+ lymphocyte loss was detected in PBMC infected with a virus with an X4 or dual tropic phenotype (Fig. 3b; NL4.3, NL4.3Δnef, or MBCD36). Furthermore, the degree of cell loss was not dramatically altered for *nef*-deleted viruses with an R5, X4, or R5X4 phenotype. Therefore, in this model of HIV-1 infection, the Nef protein did not significantly alter virus-mediated cell death.

CD4+ lymphocyte loss in primary PBMC cultures is determined by gp120 Env and is independent of viral replication kinetics

Having established that the X4 phenotype of HIV-1 causes CD4+ cell death in PBMC, irrespective of the presence of the Nef accessory protein, we next used well-characterized molecular clones NL4.3, NLAD, and AD8 to specifically define the role of virus replication and the gp120 region of Env in cell killing.

Mixed populations of PHA-PBMC were infected with an equivalent multiplicity of infection (moi) of each virus and replication kinetics, and CD4 expression was monitored over the course of the infection (Figs. 4a and c). Loss of CD4 expression in PBMC infected with NL4.3 occurred coincident with virus replication between days 5 and 10 postinfection. At the time of peak virus replication, day 11 postinfection, the majority of CD4 expression had been lost.

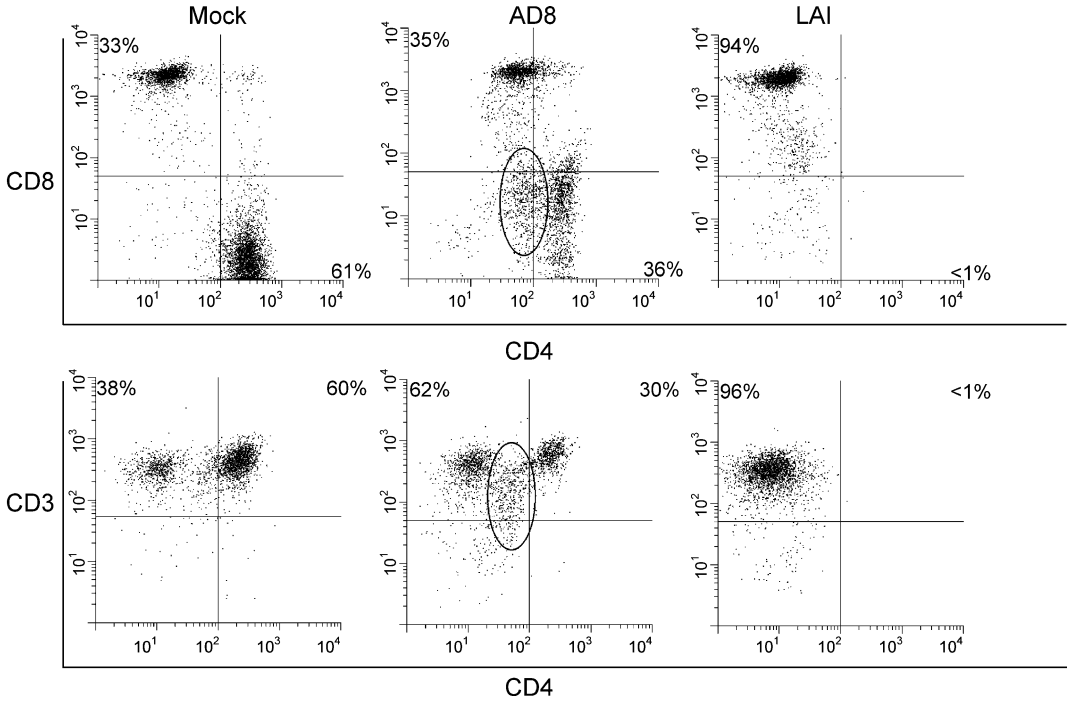

Fig. 2. Evidence of CD4 receptor downmodulation in PBMC infected with primary HIV-1 isolates. Flow cytometry analysis of a mixed population of PHA-PBMC 14 days after infection with LAI or AD8. Dual fluorescence labeling with anti-CD4-FITC/anti-CD8-PE and anti-CD4-FITC/anti-CD3-PE antibodies identified a population of CD3+ cells (circled) in the AD8-infected PBMCs that were not CD4+ or CD8+. This CD8− and CD3+/CD4− population represents PBMC in which the CD4 receptor has been downregulated. This population of cells was not detected in day 14 LAI infected PBMC, although receptor downregulation was observed earlier in PBMC infected with X4 virus (Table 1).

The replication kinetics of NLAD and AD8 was delayed relative to NL4.3 and was associated with a less dramatic loss of CD4 expression.

It has been suggested that the acute nature of the replication kinetics of the X4 viral phenotype may be responsible for the rapid cell killing by this virus phenotype relative to an R5 phenotype (Zhang et al., 1997). To determine whether CD4 cell loss in NL4.3 virus-infected cultures was a function of replication kinetics or an intrinsic function of Env, donor PBMC populations were screened to identify preferred donor populations in which all three HIV-1 virus phenotypes replicated with equivalent efficiency. Synchronization of the cell-population was further used to enhance virus replication. In contrast to previous studies (Penn et al., 1999), this approach increased the infectivity of the R5 viral phenotype and resulted in near equivalent replication of the three virus phenotypes over the acute phase of infection, days 2–6 postinfection (Fig. 4b). Characterization of the cell populations that supported equivalent replication of both the R5 and X4 viral phenotype indicated that the preferred populations expressed high levels of CD4 and CXCR4 and low levels of CCR5 (data not shown). This is typical for resting and PHA-stimulated PBMC after culture for several days in IL-2 (Bleul et al., 1997; Chanel et al., 2002; Platt et al., 1998). Although CCR5 expression on PBMC has been shown to correlate with susceptibility to infection (Wu et al., 1997), relatively low levels of CCR5 (10%) and high levels of CXCR4 (70%) on anti-CD3/IL-2-stimulated PBMC have been shown to support similar levels of R5 and X4 virus infection (Wu et al., 1997).

In all HIV-1-infected cultures, there was a loss of CD4 expression 2–3 days postinfection, which coincided with virus production (Fig. 4d). However, CD4 expression continued to decline rapidly in NL4.3-infected cells over the exponential phase of virus production, whereas the rate of CD4 loss in NLAD- and AD8-infected cultures was less dramatic. Interestingly, the rapid replication kinetics exhibited by NLAD and AD8 in the preferred synchronized PHA-PBMC population was also associated with an increased loss of CD4 expression when compared to that of the mixed PBMC culture (compare Figs. 4c and d). Nonetheless, the cytopathicity of the X4 virus was greater than that of the R5 virus phenotype despite equivalent replication kinetics, thus confirming that CD4 cell loss in HIV-1-infected cultures is not a function of virus replication (Penn et al., 1999), and indicating that Env, in particular the gp120 region, is a sole mediator of X4-virus-mediated cell loss.

Apoptosis is not detected in whole cell population of primary PBMC infected with HIV-1

Both apoptosis and necrosis have been implicated as mechanisms of HIV-1-induced cell death. As this may reflect a temporal difference in virus replication, PBMC cultures were sampled daily over the lag and exponential phases of replication and at the peak of virus production to

Table 1
The CD4 receptor is downmodulated in HIV-1-infected PBMC

Isolate (Day PI)	CD4 − /CD8 +	CD4 − /CD3 +
LAI (4)	31.49	70.70
LAI (7)	63.78	78.46
LAI (14)[a]	94.11	96.21
MBC200 (4)	40.32	55.13
MBC200 (7)	66.73	78.48
MBC200 (14)	86.80	94.86
AD8 (4)	32.05	44.51
AD8 (7)	30.09	46.46
AD8 (14)[a]	35.24	62.37
MBC676 (4)	32.04	41.41
MBC676 (7)	36.66	52.15
MBC676 (14)	49.26	75.78
MBC925 (4)	29.80	44.08
MBC925 (7)	30.08	54.67
MBC925 (14)	37.38	62.31
Mock (4)	34.20	43.09
Mock (7)	30.56	31.73
Mock (14)[a]	32.73	38.47

Flow cytometry analysis of HIV-1-infected PBMC by dual fluorescence labeling with anti-CD4-FITC/anti-CD8-PE and anti-CD4-FITC/anti-CD3-PE antibodies indicated that the CD4 receptor was initially downmodulated following infection with HIV-1. CD4 downmodulation is represented by a CD4 − /CD3+ population that cannot be attributed to the CD4 − /CD8+ population of cells. CD4 downmodulation was followed by CD4+ lymphocyte loss in PBMC infected with virus with an X4 phenotype (LAI and MBC200) but not cells infected with virus with an R5 phenotype (AD8, MBC676, and MBC925).

[a] Flow cytometry data of PBMC infected with LAI, AD8, and mock-infected PBMC are shown in Fig. 2.

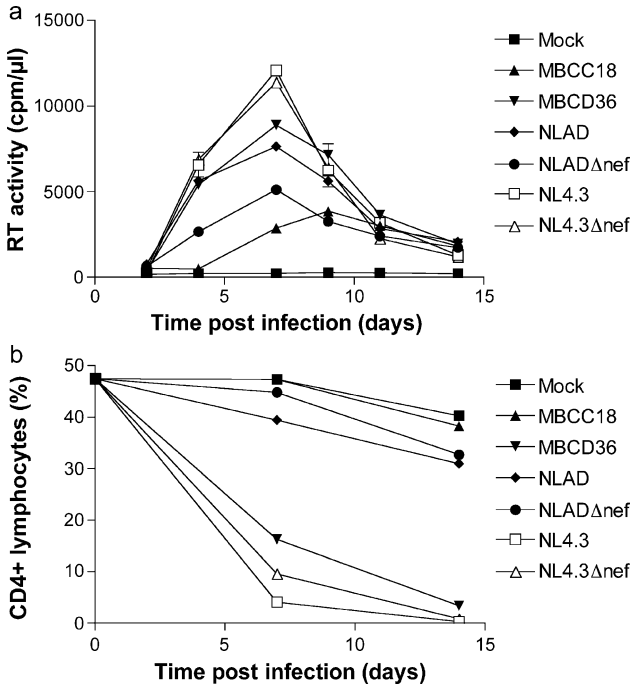

Fig. 3. The absence of the *nef* gene product does not exacerbate CD4+ lymphocyte loss in HIV-1-infected PBMC. PHA-PBMCs were mock-infected or infected with MBCC18, MBCD36, NLAD, NLADΔnef, NL4.3, or NL4.3Δnef at an moi of 0.1. Virus replication was determined by cell-free RT activity (a) and CD4+ lymphocyte loss was determined by flow cytometry analysis of cells labeled with an anti-CD4-PE antibody (b). This figure is representative of three infections using different PHA-PBMC populations.

determine whether apoptosis was responsible for the decline in CD4+ lymphocytes observed in our acute model of virus replication. Apoptosis was measured using TUNEL (Fig. 5a) and results confirmed using propidium iodide staining for cells in the hypodiploid fraction of the cell cycle (Fig. 5b). Within 24 h of culture, both mock- and virus-infected PBMCs underwent a modest increase in apoptosis. This was most likely the result of the synchronization process and culture conditions in vitro (McGahon et al., 1995). Within 48 h, the level of apoptosis had returned to preinfection levels indicating the transient nature of this effect. Over the remaining period, there was no significant increase in apoptosis in virus-infected cultures relative to mock-infected cells. There was a small decrease in the viability of cells, as determined by exclusion of the vital dye trypan blue, in virus-infected cultures relative to mock-infected cultures (Fig. 5c). However, there

Table 2
Co-receptor usage by primary and reference HIV-1

	Co-receptor usage										
	CD4 only	CCR2b	CCR3	CCR5	CCR8	CXCR4	CX3CR1	Gpr1	Gpr15	Strl33	Apj
NL4.3	−	−	−	−	−	+++	−	−	−	−	+
NLAD	−	−	++	+++	+	−	+/−	−	+	+	+
AD8	−	−	++	+++	+/−	−	−	−	+	+	+
89.6	−	+	++	+++	−	+++	−	+/−	−	−	+
MBCD36	−	+/−	−	+++	−	+++	−	−	−	−	−

Co-receptor usage of HIV-1 was determined using Cf2-Luc cells as previously described (Gorry et al., 2001, 2002a, 2002b). Cf2-Luc cells (Etemad-Moghadam et al., 2000) are derived from the Cf2th canine thymocyte cell line (Choe et al., 1996), and stably express the luciferase gene under the control of the HIV-1 LTR. Cf2-Luc cells were transfected with a plasmid expressing CD4 only as a negative control, or co-transfected with plasmids expressing CD4 and an alternative HIV-1 co-receptor and infected with equivalent amounts of each HIV-1 virus. After overnight infection, the virus was removed and the cells were cultured for an additional 48 h. Cell lysates were prepared and assayed for luciferase activity (Promega) according to the manufacturer's protocol. Entry levels were scored as +++ (>50,000 luciferase activity units), ++ (between 30,000 and 50,000 luciferase activity units), + (between 10,000 and 30,000 luciferase activity units), +/− (between 5000 and 10,000 luciferase activity units), or − (< 5000 luciferase activity units) as previously described (Gorry et al., 2001). Isolate 89.6 has been included as an example of a dual tropic virus.

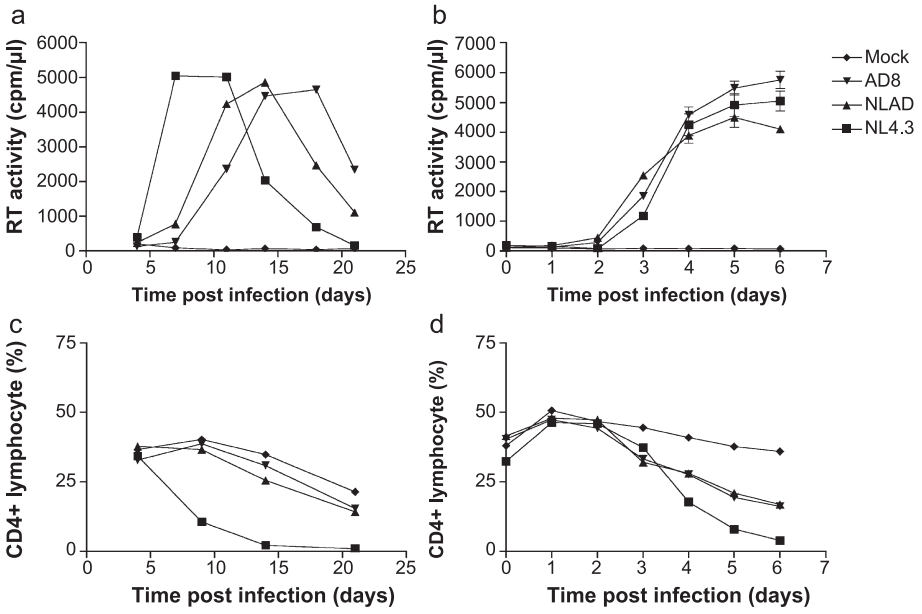

Fig. 4. Virus replication and CD4 antigen expression on HIV-1-infected PBMC. Cell-free RT activity (a and b) and CD4+ lymphocyte depletion (c and d) in NL4.3, NLAD, AD8, or mock-infected mixed PHA-PBMC population infected with a moi of 0.0001 (a and c) or a synchronized preferred PHA-PBMC population infected with a moi of 0.1 (b and d). The percentage of CD4+ lymphocytes was determined by flow cytometry analysis of cells labeled with an anti-CD4-PE antibody. Results (a and c) are representative of infections using different mixed PHA-PBMC populations. Results (b and d) are representative of three independent infections using the same donor cell population.

was no difference between the cell viability of PBMC infected with the R5 and X4 HIV-1 virus strains.

Apoptosis is detected in CD4+ X4 virus-infected cells after the exponential phase of virus replication

Although apoptosis was not detected in whole PBMC cultures infected with HIV-1, it has been reported to occur in both HIV-1-infected and -uninfected CD4+ lymphocytes as well as in uninfected CD8+ lymphocytes (Clerici et al., 1996; Finkel et al., 1995; Gougeon et al., 1996; Herbein et al., 1998a, 1998b; Meyaard et al., 1992; Muro-Cacho et al., 1995). To investigate apoptosis in specific cell subsets, we undertook dual fluorescence labeling with the TUNEL assay and fluorescence labeled anti-CD4 or anti-p24 antibodies. Labeling of cells for intracellular p24 expression indicated that only a small percentage of the population was infected. No more than 5% of NL4.3-infected PBMCs were p24 positive, whereas as many as 7% of NLAD- and 13% of AD8-infected cells were p24-positive during the exponential phase of virus replication. Dual labeling with TUNEL did not identify a correlation between apoptosis and antigen expression in infected cells or apoptosis in the uninfected bystander population (Fig. 6).

Dual labeling for expression of CD4 and apoptosis indicated that a similar percentage of $CD4^+$ cells were undergoing apoptosis in both HIV-1-infected and mock-infected cell cultures (Figs. 6 and 7a). However, a significant proportion of CD4+ lymphocytes was undergoing apoptosis in NL4.3-infected cultures by 5 days postinfection, at which time the majority of CD4+ lymphocyte loss had occurred (Fig. 7b). It was unclear whether this increased level of apoptosis reflected a constant level of apoptosis in a declining cell population or an increasing level of apoptosis that was associated with HIV-1-infection and masked by the outgrowth of other cells, CD8+ lymphocytes in particular, in the whole PBMC population (data not shown). To address this question, we depleted the preferred PBMC population of CD8+ lymphocytes and repeated this acute infection with NL4.3 in either this depleted cell culture or parallel whole PBMC population.

Apoptosis was assessed in parallel infections of whole PBMC (Fig. 8a) and CD8-depleted cultures with NL4.3 (Fig. 8b) on days 2 and 6 postinfection at which time both cultures contained similar levels of cell-free RT activity (approximately 3000 cpm/µl; data not shown). Before virus replication, 2 days postinfection, there was no significant difference between the levels of apoptosis in uninfected or NL4.3-infected whole PBMC or CD8-depleted cultures. Whereas, on day 6, at the time of peak virus production, there was an almost two-fold increase in the level of apoptosis detected in CD8-depleted cells infected with NL4.3 relative to mock-infected cells. This was in contrast with parallel infections of whole PBMC population in which there was no obvious difference between the levels of apoptosis in NL4.3- and mock-infected cultures. Thus, the increased levels of apoptosis detected in the analysis shown in Fig. 7b are most likely to represent an increased level of apoptosis associated with HIV-1 infection. Interestingly, in these experiments, increased levels of apoptosis were

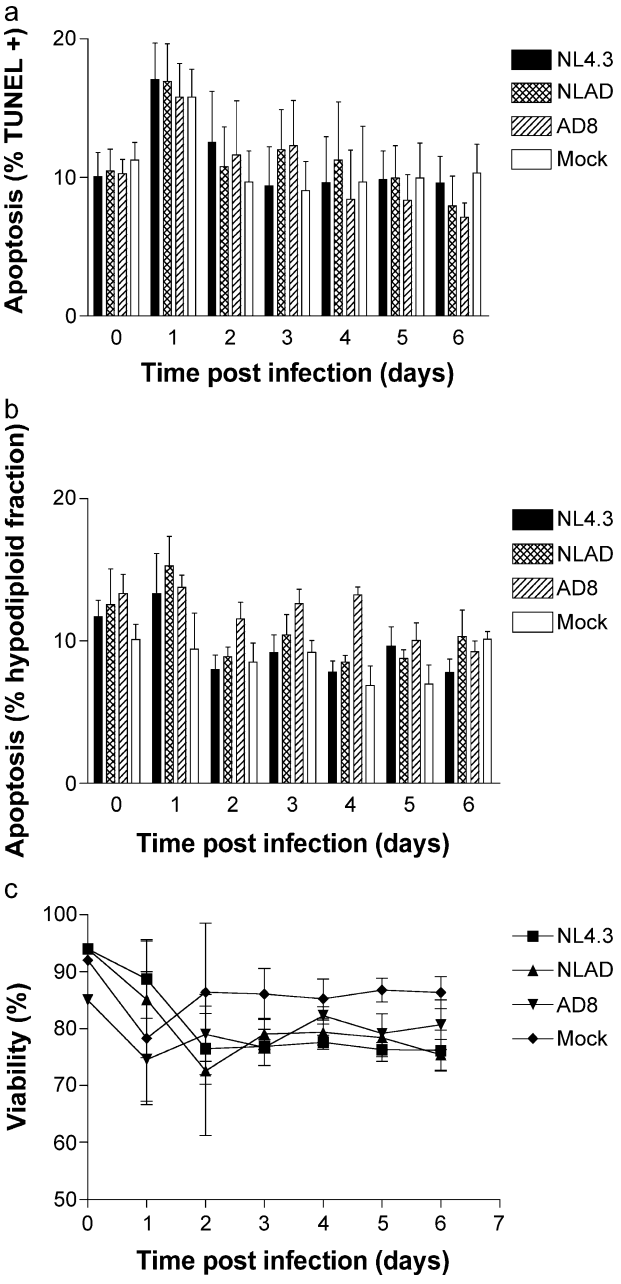

Fig. 5. Mechanisms of cell death in PBMC infected with HIV-1. Synchronized preferred PHA-PBMCs were mock infected or infected with NL4.3, NLAD, or AD8 at a moi of 0.1. Apoptosis was detected by fluorescein-conjugated TUNEL (a) or the hypodiploid fraction following propidium iodide staining for cell cycle analysis (b). Viable cells were detected by exclusion of the viable dye trypan blue (c). Results are the average of three independent infections of the same preferred PHA-PBMC population used in Fig. 4. Fig. 4 is representative of the replication kinetics and CD4+ lymphocyte loss observed in these infections. TUNEL and the hypodiploid peak were assayed in duplicate for each infection. TUNEL fluorescence was gated between 1% and 2% of mock-infected cells labeled without enzyme. Propidium iodide-stained cells were gated to exclude doublets and debris.

detected in the p24-negative cell population (data not shown). Thus supporting a role for bystander cell death late in the infection period.

Discussion

The variable replication of some HIV-1 isolates in primary cells derived from different donors has been described for both PBMC (Evans et al., 1987; Spira and Ho, 1995) and monocyte-derived macrophages (Cunningham et al., 2000). This phenomenon was exploited in the present study by selection of preferred PHA-PBMC populations, which supported equivalent replication by virus with either an R5 or X4 Env viral phenotypes. Replication was further enhanced by the synchronization of the population, which promoted an efficient single round of virus infection. We used this system to show that the loss of $CD4^+$ lymphocytes from primary PBMC cultures is dependent on the viral phenotype, in particular the gp120 region of HIV-1 Env, and is not dependent on viral replication kinetics during acute infection. Detailed kinetic studies indicated that apoptosis was detected after the exponential phase of virus replication in NL4-3 infected PBMC and after the majority of cell loss had occurred. Thus, in this acute model of HIV-1 infection, apoptosis is not the primary mediator of CD4+ T-lymphocyte loss.

The cytopathicity of the X4 virus NL4-3 in PBMC is attenuated when the V3 region of Env is replaced with that of an R5 virus (Penn et al., 1999). We further demonstrated that the cytopathic profile of the chimeric virus NLAD was identical to that of its host Env region AD8. Thus, establishing that Env and in particular the gp120 region is the sole mediator of cell loss observed in NL4.3-infected PBMC.

The method used to equalize X4 and R5 virus replication kinetics enhanced the replication of the R5 virus phenotype. Entry independent expression of an R5 Env has been shown to effectively cause cytolysis in a membrane fusion-dependent manner (LaBonte et al., 2000). Interestingly, cell fusion was observed in AD8-infected cultures of synchronized preferred PBMC (data not shown) and the level of CD4 expression in these cultures was somewhat decreased relative to a mixed PBMC population. Thus suggesting that the R5-induced cytopathicity may have been enhanced under the conditions used here. However, the R5 virus phenotype was unable to attain the levels of cell loss detected in X4 virus-infected PBMC, thus supporting an intrinsic cytopathicity associated with an X4 Env that is independent of virus replication.

In this and a previous study, low levels of apoptosis were detected in CD4+ lymphocytes of HIV-1-infected PBMC at the peak of virus replication (Herbein et al., 1998a), but after the majority of CD4+ lymphocyte loss has occurred (Fig. 8b). Thus, supporting the view that apoptosis occurs as a consequence of CD4 + lymphocyte loss that is mediated by a non-apoptotic mechanism. Apoptosis remains an important mechanism of bystander cell loss in HIV-1 infection and requires the presence of macrophages (Herbein et al., 1998a, 1998b). Despite the presence of macrophages in our primary cell cultures, we did not consistently detect apoptosis in bystander cells of the whole PBMC infections. However, in

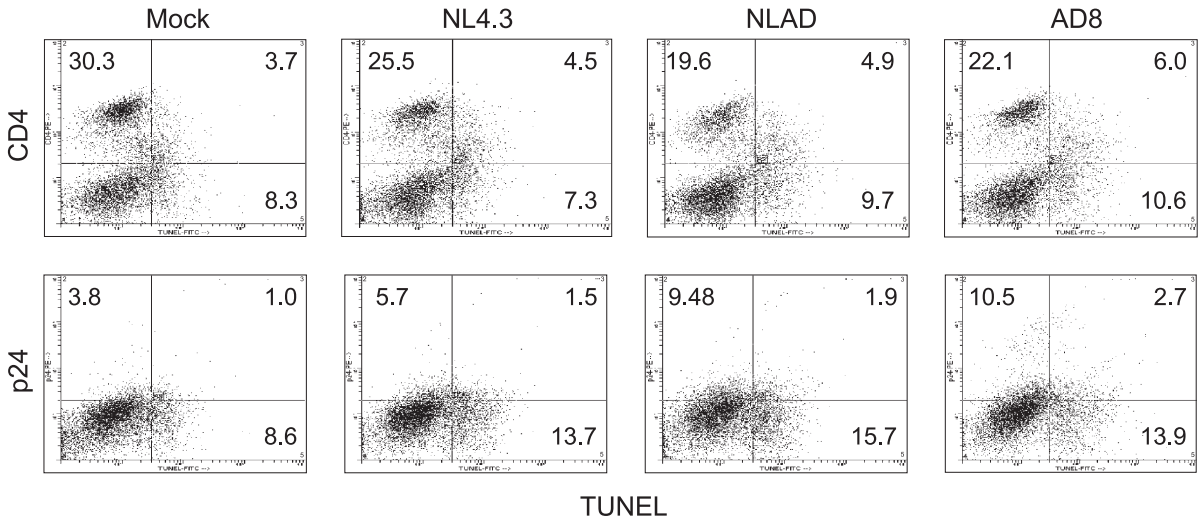

Fig. 6. Apoptosis is not detected in the bystander cell population of HIV-1-infected PBMC during the acute phase of virus replication. Representative flow cytometry analysis of NL4.3, NLAD, AD8, or mock-infected synchronized preferred PHA-PBMC. Dual fluorescence labeling for whole cell CD4 expression (anti-CD4-PE) or viral antigen (anti-p24-PE) was performed in conjunction with fluorescein-conjugated TUNEL (TUNEL-FITC) to identify in which cell population apoptosis was occurring. The phase of exponential virus replication and cell loss is shown (3 days postinfection).

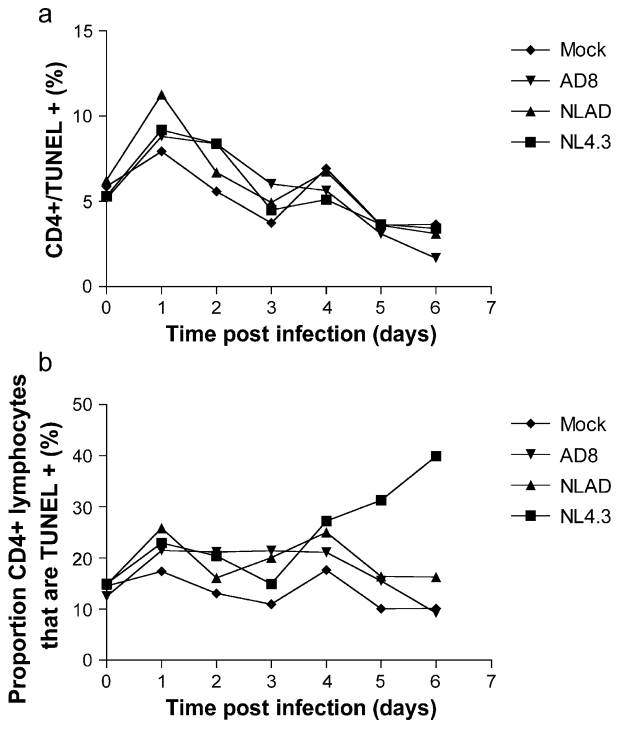

Fig. 7. Apoptosis in NL4.3-infected PBMC is detected after CD4+ lymphocyte loss. Synchronized preferred PHA-PBMCs were infected with NL4.3 (ν), NLAD (σ), or AD8 (τ) at a moi of 0.1 or mock-infected (υ), and dual fluorescence labeled for whole cell CD4 expression (anti-CD4-PE) and fluorescein-conjugated TUNEL (TUNEL-FITC) to identify in which cell population apoptosis was occurring. From flow cytometry analysis of the percentage of apoptotic CD4+ (CD4+/TUNEL+) cells in the whole population (a) and the proportion of CD4+ cells that undergo apoptosis (TUNEL+) (b) was determined. Result shown is representative of three independent infections of the same preferred PHA-PBMC population used in Fig. 4. Fig. 4 is representative of the replication kinetics and CD4+ lymphocyte loss observed in these infections.

this study, the period of observation did not extend beyond the peak of virus replication when bystander cell death has previously been detected.

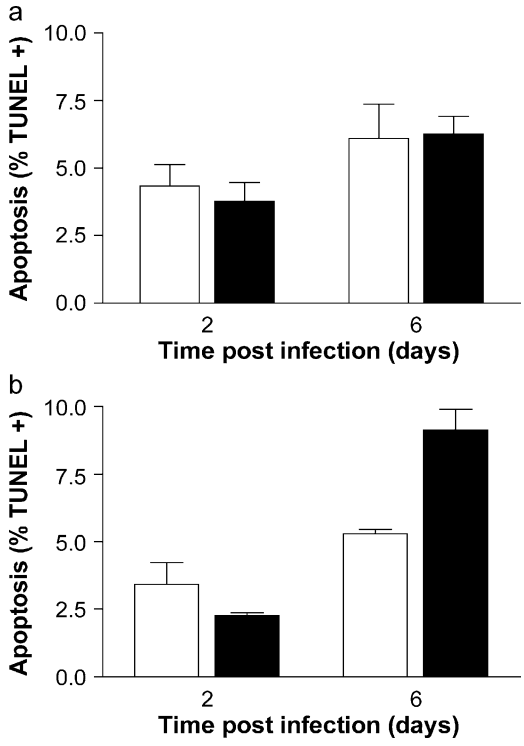

Fig. 8. Apoptosis is detected in NL4.3-infected CD8-depleted PBMC. Apoptosis was detected by fluorescein-conjugated TUNEL assay of whole (a) or CD8+-depleted (b) synchronized PHA-PBMC infected with NL4.3 (solid columns) at a moi of 0.1 or mock-infected (open columns). Example shown is representative of three independent infections using two different preferred PHA-PBMC populations; error bars represent the standard error in duplicate samples assayed within the same infection.

This study has implications in relation to the course of disease in HIV-1 infection. The initial infection process in vivo is almost exclusively by R5 viruses. Although we show here that R5 viruses have lower cytopathicity for PBMC than X4 viruses, it has been proposed that the high turnover rate of memory T-cells that express CCR5 in vivo would lead to high levels of R5 virus infection and cell death over time (Davenport et al., 2002). This would eventually lead to a decline in CD4+ T-lymphocyte numbers and the activation of naive T-lymphocytes that are susceptible to infection with more cytopathic X4 virus types. Infection and death of these cells would then induce apoptosis as described here and in bystander CD8+ T-lymphocytes (Herbein et al., 1998a; Zaunders et al., 2003). However, the initial CD4+ lymphocyte loss is not mediated by apoptosis but most probably by necrosis (Arnoult et al., 2003; Bolton et al., 2002; Borthwick et al., 1999; Kolesnitchenko et al., 1995, 1997; LaBonte et al., 2000; Plymale et al., 1999). Certainly, apoptosis may be occurring in infected cells, predominantly in association with the X4 virus phenotype, but only because of virus infection (Herbein et al., 1998b; Kreisberg et al., 2001; Lenardo et al., 2002).

Our results indicate that the HIV-1 Nef protein is not making a major contribution to cell death. The involvement of HIV-1 Nef in controlling cell-mediated apoptosis remains controversial (Greenway et al., 2002, 2003), but importantly, within the context of a viral infection, there is potential interplay among all the HIV-1 proteins. Of particular note are Tat, Nef, and Vpr, all of which have been identified as inducing or preventing apoptosis (Arnoult et al., 2003; Greenway et al., 2002; Waldhuber et al., 2003).

Our results predict a temporal role for both apoptosis-independent and -dependent HIV-1-mediated cell killing. Early during the exponential phase of virus replication in cells infected with an X4 virus, cell death occurs independently of apoptosis. But at the peak of virus production, further cell killing occurs via apoptotic mechanisms. This two-phase cell killing has been observed in LAI-infected Jurkat cells in which cell death is not attenuated by the presence of Bcl-2 (an inhibitor of apoptosis) until the peak of virus replication has been reached (Kolesnitchenko et al., 1997). This may represent an early apoptosis-independent, virus replication-dependent form of cell death followed by replication-independent apoptosis, which is consistent with the apoptosis observed in bystander cells of HIV-1-infected individuals (Clerici et al., 1996; Finkel et al., 1995; Gougeon et al., 1996; Herbein et al., 1998b; Meyaard et al., 1992; Muro-Cacho et al., 1995).

Materials and methods

Preparation of primary cells

HIV-1-negative PBMCs were isolated by density gradient centrifugation over Ficoll hypaque according to the method of Neate et al. (1987). Isolated cells were either phytohemagglutinin activated for infection (PHA-PBMC) or stored in liquid nitrogen for subsequent screening of their ability to support the replication of X4 and R5 HIV-1 phenotypes.

Cells were PHA activated for 72 h in RPMI 1640 medium (ICN) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (0.2 mM) (RF10), and PHA (10 μg/ml; Murex Biotech) before infection. These PHA-PBMCs were then maintained in interleukin-2 (IL-2) medium [RF10 containing IL-2 (10 U/ml; Boehringer Mannheim), polybrene (2 μg/ml), and hydrocortisone (4 μg/ml)] at 1×10^6 cells/ml.

Preferred populations of PHA-PBMCs were selected for their ability to support equivalent replication for both X4 and R5 viruses at a multiplicity of infection (moi) of 0.1–10. These cells were synchronized by exposure to IL-2 (10 U/ml in IL-2 medium) for 6 h followed by incubation in RF10 alone for 48 h to accumulate cells in G_0/G_1 phase of the cell cycle as previously described (Stern and Smith, 1986). Synchronized PHA-PBMCs were then maintained in IL-2 medium.

PHA-PBMCs were enriched for CD4+ lymphocytes by depletion of CD8-expressing cells using M-450 CD8 Dynabeads according to the manufacturer's instructions (Dynal). After removal of CD8+ cells, the cell suspension was pelleted and resuspended in IL-2 medium for infection. Flow cytometric analysis indicated that after CD8 depletion, more than 70% of the population was CD4+ and less than 10% of the population was CD8+ (data not shown).

Viral replication kinetics were determined in mixed (two donor populations) PHA-PBMCs infected with an equivalent moi from virus stocks. The mixed PHA-PBMC population was incubated with virus stocks for 2 h at 37 °C, washed twice in PBS, and then resuspended in IL-2 medium at a concentration of 1×10^6/ml. Cell suspensions (200 μl) were set up in duplicate wells of 96-well plates. Cultures were half medium changed twice weekly for 4 weeks and the supernatant stored at −20 °C to determine cell-free reverse transcriptase (RT) activity. Cultures were supplemented weekly with an equal number of fresh donor PHA-PBMC.

Virus stocks

Infectious stocks of the molecular clones of LAI (Peden et al., 1991), NL4-3 (Adachi et al., 1986), AD8 (Theodore et al., 1996), and the chimeric clone NLAD (Freed et al., 1994), and the *nef*-deleted clones NL4-3Δnef and NLAD Δnef (Gibbs et al., 1994) were obtained from the Australian National Centre in HIV Virology research (NCHVR) Research Reagent program.

Australian HIV-1 isolates MBC200, MBC925, and MBC676 were isolated from PBMC, CSF, and brain, respectively, and were kindly provided by Ms. Sally Land

(State HIV Isolation Laboratory, Victoria, Australia) (Cameron et al., 1996; Kiernan et al., 1990; Oelrichs et al., 2000). Primary isolates MBCD36 (X4R5) and MBCC18 (R5) from the Sydney blood bank cohort (SBBC) (Deacon et al., 1995) were both attenuated in nef/LTR (McPhee, D, unpublished). Stocks were generated by a single passage in a mixed PHA-PBMC population.

High titer stocks of virus from the HIV-1 molecular clones NL4.3, NLAD, and AD8 were prepared from day 14 supernatants of infected PHA-PBMC. The supernatant was clarified by low speed centrifugation at 146 × *g* for 4 min and virus concentrated at 72000 × *g* for 1 h. The supernatant was discarded and the viral pellets were resuspended in IL-2 medium to one-tenth original volume. The titer of virus obtained by this method was between 1×10^5 and 1×10^6 $TCID_{50}$/ml.

Co-receptor usage

The co-receptor usage of each virus was determined using Cf2-Luc cells as previously described (Gorry et al., 2001, 2002a, 2002b). Cf2-Luc cells (Etemad-Moghadam et al., 2000) are derived from the Cf2th canine thymocyte cell line (Choe et al., 1996) and stably express the luciferase gene under the control of the HIV-1 LTR. Cf2-Luc cells were transfected with a plasmid expressing CD4 only as a negative control or co-transfected with plasmids expressing CD4 and an alternative HIV-1 co-receptor and infected with equivalent amounts of each HIV-1 virus. After overnight infection, the virus was removed and the cells were cultured for an additional 48 h. Cell lysates were prepared and assayed for luciferase activity (Promega) according to the manufacturer's protocol.

Virus replication

Cell-free HIV-1 RT activity was detected with a modified micro-RT assay (Willey et al., 1988) using ^{33}P instead of ^{32}P and read on a 1450 Microbeta Plus liquid scintillation counter (Wallac). Infection supernatant was considered positive if the RT activity was at least twice that of the uninfected control supernatant.

Flow cytometric analysis

Samples were analyzed on a FACStar Plus flow cytometer (Becton Dickinson) using an Innova laser (Coherent Scientific) at the wavelength of 488 nm and output power of 200 mW. At least 10,000 events of interest were captured per sample and the lymphocyte population was gated by forward scatter (FSC) and side scatter (SSC) profiles. Data were analyzed using the Winlist suite of programs (Verity Software House, Version 2.0) on a PC platform.

Fluorescence-conjugated monoclonal antibodies (Becton Dickinson) were used to detect the surface expression of PBMC antigens (CD4, CD8, and CD3). Cells were washed with 2.5% (v/v) FCS/0.1% (w/v) NaN_3/PBS before the addition of 10 μl of antibody and incubated on ice for 45–60 min. The cells were washed before being fixed in 1.0% (v/v) formaldehyde/PBS for analysis. Specific fluorescence was gated relative to 5% of an isotype control antibody.

To detect intracellular HIV-1 core antigen (p24), the cells were washed and fixed on ice for 45 min in 4% (w/v) paraformaldehyde (PFA; Merck)/PBS, pH 7.4. Free aldehydes were quenched with 0.1 M glycine/0.1% (w/v) NaN_3/PBS and permeabilized by treatment with 0.1% (v/v) Triton X-100/PBS for 1 min. The fixed and permeabilized cells were incubated on ice for 1 h with an anti-p24-specific monoclonal antibody (2 μg/ml; Olympus Corporation), 5% (v/v) FCS/0.1% (w/v) NaN_3/PBS followed by a secondary fluorescein-conjugated anti-mouse antibody (Silenus). The labeled cells were fixed in 1.0% (v/v) formaldehyde/PBS and analyzed. Specific fluorescence was gated relative to 5% of mock-infected cells labeled with anti-p24 antibody.

Detection of apoptosis

Apoptotic cells have a reduced DNA content, which can be detected as reduced fluorescence in flow cytometric analysis of cell cycle using the dye propidium iodide. Briefly, cells were washed once with PBS and fixed on ice for 1 h in 1 ml 0.5% (v/v) formaldehyde/PBS. The cells were washed in PBS, resuspended in 500 μl of propidium iodide stain solution (3%(w/v) PEG, 50 μg/ml propidium iodide, 10 μg/ml Dnase-free Rnase, 0.1% (v/v) TX-100 in 4 mM citric acid pH 7.8), and incubated for 20 min at 37 °C. The cells were then left overnight at 4 °C after the addition of 500 μl propidium iodide salt solution (3%(w/v) PEG, 50 μg/ml propidium iodide, 0.1% (v/v) TX-100 in 0.4 mM NaCl) and then analyzed. The apoptotic cell fraction was gated below the G_0/G_1 peak of the cell cycle of the control cell population with gating at channel 10 of the fluorescence scale to eliminate debris.

Cleavage of nuclear DNA into oligonucleosome-sized fragments was detected in individual cells using the fluorescein-conjugated in situ cell death detection kit according to the manufacturer's instructions (Boehringer Mannheim). This kit detects DNA cleavage by the TUNEL method, which labels the blunt ends of double-stranded DNA breaks with a fluorescein-labeled d-UTP using a terminal deoxynucleotidyl transferase enzyme. Briefly, 0.2 or 1.0×10^6 cells were washed twice in 2–3 ml 1% (w/v) BSA/PBS. The final pellet was resuspended in 1.0 ml freshly prepared 4% (w/v) PFA/PBS, pH 7.4, and incubated at room temperature for 30 min. Cells were washed once in 1% (w/v) BSA/PBS, resuspended, and vortexed in 1.0 ml of 0.1% (v/v) TX-100/PBS for 1 min. Thus permeabilized, the cells were immediately washed twice in 1%(w/v) BSA/PBS and resuspended in 50 μl TUNEL reaction mixture and then incubated for 1 h at 37 °C in a darkened, humidified atmosphere. The cells were washed a final two times in

1%(w/v) BSA/PBS and resuspended in 1% (v/v) formaldehyde/PBS and stored at 4 °C until analyzed.

Apoptosis was detected in the CD4+ lymphocyte population or HIV-1-infected cells by dual labeling for whole cell CD4 or intracellular p24 antigen expression in conjunction with TUNEL. Intracellular p24 antigen expression was detected in fixed, permeabilized cells and labeled as described above. Whole cell CD4 expression was detected by preparing cells as described for intracellular p24 expression and substituting the anti-CD4-PE-conjugated antibody for the primary anti-p24 antibody, secondary labeling with the fluorescein-conjugated antibody was omitted. Before final fixation in 1% (v/v) formaldehyde/PBS, the anti-p24 or anti-CD4 antibody labeled cells were washed once in 1% (w/v) BSA/PBS and resuspended in 50 μl of TUNEL reaction mixture and processed as described above for TUNEL staining.

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References

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., Martin, M.A., 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. 59 (2), 284–291.

Akari, H., Bour, S., Kao, S., Adachi, A., Strebel, K., 2001. The human immunodeficiency virus type 1 accessory protein Vpu induces apoptosis by suppressing the nuclear factor kappaB-dependent expression of antiapoptotic factors. J. Exp. Med. 194 (9), 1299–1311.

Arnoult, D., Petit, F., Lelievre, J.D., Estaquier, J., 2003. Mitochondria in HIV-1-induced apoptosis. Biochem. Biophys. Res. Commun. 304 (3), 561–574.

Benedict, C.A., Norris, P.S., Ware, C.F., 2002. To kill or be killed: viral evasion of apoptosis. Nat. Immunol. 3 (11), 1013–1018.

Bleul, C.C., Wu, L., Hoxie, J.A., Springer, T.A., Mackay, C.R., 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 94 (5), 1925–1930.

Bolton, D.L., Hahn, B.I., Park, E.A., Lehnhoff, L.L., Hornung, F., Lenardo, M.J., 2002. Death of CD4 (+) T-cell lines caused by human immunodeficiency virus type 1 does not depend on caspases or apoptosis. J. Virol. 76 (10), 5094–5107.

Borthwick, N.J., Wickremasinghe, R.G., Lewin, J., Fairbanks, L.D., Bofill, M., 1999. Activation-associated necrosis in human immunodeficiency virus infection. J. Infect. Dis. 179 (2), 352–360.

Cameron, P.U., Lowe, M.G., Sotzik, F., Coughlan, A.F., Crowe, S.M., Shortman, K., 1996. The interaction of macrophage and non-macrophage tropic isolates of HIV-1 with thymic and tonsillar dendritic cells in vitro. J. Exp. Med. 183 (4), 1851–1856.

Casella, C.R., Rapaport, E.L., Finkel, T.H., 1999. Vpu increases susceptibility of human immunodeficiency virus type 1-infected cells to fas killing. J. Virol. 73 (1), 92–100.

Chanel, C., Staropoli, I., Baleux, F., Amara, A., Valenzuela-Fernandez, A., Virelizier, J.L., Arenzana-Seisdedos, F., Altmeyer, R., 2002. Low levels of co-receptor CCR5 are sufficient to permit HIV envelope-mediated fusion with resting CD4 T cells. AIDS 16 (17), 2337–2340.

Chen, B.K., Gandhi, R.T., Baltimore, D., 1996. CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1 involves independent activities of vpu, env, and nef. J. Virol. 70 (9), 6044–6053.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., Sodroski, J., 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 85 (7), 1135–1148.

Clerici, M., Sarin, A., Berzofsky, J.A., Landay, A.L., Kessler, H.A., Hashemi, F., Hendrix, C.W., Blatt, S.P., Rusnak, J., Dolan, M.J., Coffman, R.L., Henkart, P.A., Shearer, G.M., 1996. Antigen-stimulated apoptotic T-cell death in HIV infection is selective for CD4+ T cells, modulated by cytokines and effected by lymphotoxin. AIDS 10 (6), 603–611.

Cunningham, A.L., Li, S., Juarez, J., Lynch, G., Alali, M., Naif, H., 2000. The level of HIV infection of macrophages is determined by interaction of viral and host cell genotypes. J. Leukoc. Biol. 68 (3), 311–317.

Davenport, M.P., Zaunders, J.J., Hazenberg, M.D., Schuitemaker, H., van Rij, R.P., 2002. Cell turnover and cell tropism in HIV-1 infection. Trends Microbiol. 10 (6), 275–278.

Deacon, N.J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D.J., McPhee, D.A., Greenway, A.L., Ellett, A., Chatfield, C., et al., 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science 270 (5238), 988–991.

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 (9), 4433–4440.

Evans, L.A., McHugh, T.M., Stites, D.P., Levy, J.A., 1987. Differential ability of human immunodeficiency virus isolates to productively infect human cells. J. Immunol. 138 (10), 3415–3418.

Finkel, T.H., Tudor-Williams, G., Banda, N.K., Cotton, M.F., Curiel, T., Monks, C., Baba, T.W., Ruprecht, R.M., Kupfer, A., 1995. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat. Med. 1 (2), 129–134.

Freed, E.O., Orenstein, J.M., Buckler-White, A.J., Martin, M.A., 1994. Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. J. Virol. 68 (8), 5311–5320.

Gandhi, R.T., Chen, B.K., Straus, S.E., Dale, J.K., Lenardo, M.J., Baltimore, D., 1998. HIV-1 directly kills CD4+ T cells by a Fas-independent mechanism. J. Exp. Med. 187 (7), 1113–1122.

Garcia, J.V., Miller, A.D., 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature 350 (6318), 508–511.

Gibbs, J.S., Regier, D.A., Desrosiers, R.C., 1994. Construction and in vitro properties of HIV-1 mutants with deletions in "nonessential" genes. AIDS Res. Hum. Retroviruses 10 (4), 343–350.

Gorry, P.R., Bristol, G., Zack, J.A., Ritola, K., Swanstrom, R., Birch, C.J., Bell, J.E., Bannert, N., Crawford, K., Wang, H., Schols, D., De Clercq, E., Kunstman, K., Wolinsky, S.M., Gabuzda, D., 2001. Macrophage

tropism of human immunodeficiency virus type 1 isolates from brain and lymphoid tissues predicts neurotropism independent of coreceptor specificity. J. Virol. 75 (21), 10073–10089.

Gorry, P.R., Taylor, J., Holm, G.H., Mehle, A., Morgan, T., Cayabyab, M., Farzan, M., Wang, H., Bell, J.E., Kunstman, K., Moore, J.P., Wolinsky, S.M., Gabuzda, D., 2002a. Increased CCR5 affinity and reduced CCR5/CD4 dependence of a neurovirulent primary human immunodeficiency virus type 1 isolate. J. Virol. 76 (12), 6277–6292.

Gorry, P.R., Zhang, C., Wu, S., Kunstman, K., Trachtenberg, E., Phair, J., Wolinsky, S., Gabuzda, D., 2002b. Persistence of dual-tropic HIV-1 in an individual homozygous for the CCR5 Delta 32 allele. Lancet 359 (9320), 1832–1834.

Gougeon, M.L., 1996. Apoptosis in AIDS. Genetic control and relevance for AIDS pathogenesis. Biochem. Soc. Trans. 24 (4), 1055–1058.

Gougeon, M.L., Lecoeur, H., Dulioust, A., Enouf, M.G., Crouvoiser, M., Goujard, C., Debord, T., Montagnier, L., 1996. Programmed cell death in peripheral lymphocytes from HIV-infected persons: increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression. J. Immunol. 156 (9), 3509–3520.

Greenway, A.L., McPhee, D.A., Allen, K., Johnstone, R., Holloway, G., Mills, J., Azad, A., Sankovich, S., Lambert, P., 2002. Human immunodeficiency virus type 1 Nef binds to tumor suppressor p53 and protects cells against p53-mediated apoptosis. J. Virol. 76 (6), 2692–2702.

Greenway, A.L., Holloway, G., McPhee, D.A., Ellis, P., Cornall, A., Lidman, M., 2003. HIV-1 Nef control of cell signalling molecules: multiple strategies to promote virus replication. J. Biosci. 28 (3), 311–321.

Groux, H., Torpier, G., Monte, D., Mouton, Y., Capron, A., Ameisen, J.C., 1992. Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. J. Exp. Med. 175 (2), 331–340.

Herbein, G., Mahlknecht, U., Batliwalla, F., Gregersen, P., Pappas, T., Butler, J., O'Brien, W.A., Verdin, E., 1998a. Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. Nature 395 (6698), 189–194.

Herbein, G., Van Lint, C., Lovett, J.L., Verdin, E., 1998b. Distinct mechanisms trigger apoptosis in human immunodeficiency virus type 1-infected and in uninfected bystander T lymphocytes. J. Virol. 72 (1), 660–670.

Karmochkine, M., Parizot, C., Calvez, V., Coutellier, A., Herson, S., Debre, P., Gorochov, G., 1998. Susceptibility of peripheral blood mononuclear cells to apoptosis is correlated to plasma HIV load. J. Acquired Immune. Defic. Syndr. Hum. Retrovirol. 17 (5), 419–423.

Kestler III, H.W., Ringler, D.J., Mori, K., Panicali, D.L., Sehgal, P.K., Daniel, M.D., Desrosiers, R.C., 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell 65 (4), 651–662.

Kiernan, R., Marshall, J., Bowers, R., Doherty, R., McPhee, D., 1990. Kinetics of HIV-1 replication and intracellular accumulation of particles in HTLV-I-transformed cells. AIDS Res. Hum. Retroviruses 6 (6), 743–752.

Kirchhoff, F., Greenough, T.C., Brettler, D.B., Sullivan, J.L., Desrosiers, R.C., 1995. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N. Engl. J. Med. 332 (4), 228–232.

Kolesnitchenko, V., Wahl, L.M., Tian, H., Sunila, I., Tani, Y., Hartmann, D.P., Cossman, J., Raffeld, M., Orenstein, J., Samelson, L.E., et al., 1995. Human immunodeficiency virus 1 envelope-initiated G2-phase programmed cell death. Proc. Natl. Acad. Sci. U.S.A. 92 (25), 11889–11893.

Kolesnitchenko, V., King, L., Riva, A., Tani, Y., Korsmeyer, S.J., Cohen, D.I., 1997. A major human immunodeficiency virus type 1-initiated killing pathway distinct from apoptosis. J. Virol. 71 (12), 9753–9763.

Kreisberg, J.F., Kwa, D., Schramm, B., Trautner, V., Connor, R., Schuitemaker, H., Mullins, J.I., van't Wout, A.B., Goldsmith, M.A., 2001. Cytopathicity of human immunodeficiency virus type 1 primary isolates depends on coreceptor usage and not patient disease status. J. Virol. 75 (18), 8842–8847.

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 (22), 10690–10698.

Laurent-Crawford, A.G., Krust, B., Riviere, Y., Desgranges, C., Muller, S., Kieny, M.P., Dauguet, C., Hovanessian, A.G., 1993. Membrane expression of HIV envelope glycoproteins triggers apoptosis in CD4 cells. AIDS Res. Hum. Retroviruses 9 (8), 761–773.

Laurent-Crawford, A.G., Coccia, E., Krust, B., Hovanessian, A.G., 1995. Membrane-expressed HIV envelope glycoprotein heterodimer is a powerful inducer of cell death in uninfected CD4+ target cells. Res. Virol. 146 (1), 5–17.

Lenardo, M.J., Angleman, S.B., Bounkeua, V., Dimas, J., Duvall, M.G., Graubard, M.B., Hornung, F., Selkirk, M.C., Speirs, C.K., Trageser, C., Orenstein, J.O., Bolton, D.L., 2002. Cytopathic killing of peripheral blood CD4 (+) T lymphocytes by human immunodeficiency virus type 1 appears necrotic rather than apoptotic and does not require env. J Virol 76 (10), 5082–5093.

Mahlknecht, U., Deng, C., Lu, M.C., Greenough, T.C., Sullivan, J.L., O'Brien, W.A., Herbein, G., 2000. Resistance to apoptosis in HIV-infected CD4+ T lymphocytes is mediated by macrophages: role for Nef and immune activation in viral persistence. J. Immunol. 165 (11), 6437–6446.

McGahon, A.J., Martin, S.J., Bissonnette, R.P., Mahboubi, A., Shi, Y., Mogil, R.J., Nishioka, W.K., Green, D.R., 1995. The end of the (cell) line: methods for the study of apoptosis in vitro. Methods Cell Biol. 46, 153–185.

Meyaard, L., Otto, S.A., Jonker, R.R., Mijnster, M.J., Keet, R.P., Miedema, F., 1992. Programmed death of T cells in HIV-1 infection. Science 257 (5067), 217–219.

Muro-Cacho, C.A., Pantaleo, G., Fauci, A.S., 1995. Analysis of apoptosis in lymph nodes of HIV-infected persons. Intensity of apoptosis correlates with the general state of activation of the lymphoid tissue and not with stage of disease or viral burden. J. Immunol. 154 (10), 5555–5566.

Neate, E.V., Pringle, R.C., Jowett, J.B., Healey, D.S., Gust, I.D., 1987. Isolation of HIV from Australian patients with AIDS, AIDS related conditions and healthy antibody positive individuals. Aust. N. Z. J. Med. 17 (5), 461–466.

Oelrichs, R.B., Lawson, V.A., Coates, K.M., Chatfield, C., Deacon, N.J., McPhee, D.A., 2000. Rapid full-length genomic sequencing of two cytopathically heterogeneous Australian primary HIV-1 isolates. J. Biomed. Sci. 7 (2), 128–135.

Oyaizu, N., McCloskey, T.W., Coronesi, M., Chirmule, N., Kalyanaraman, V.S., Pahwa, S., 1993. Accelerated apoptosis in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type-1 infected patients and in CD4 cross-linked PBMCs from normal individuals. Blood 82 (11), 3392–3400.

Oyaizu, N., McCloskey, T.W., Than, S., Hu, R., Pahwa, S., 1995. Mechanism of apoptosis in peripheral blood mononuclear cells of HIV-infected patients. Adv. Exp. Med. Biol. 374, 101–114.

Pandolfi, F., Pierdominici, M., Oliva, A., D'Offizi, G., Mezzaroma, I., Mollicone, B., Giovannetti, A., Rainaldi, L., Quinti, I., Aiuti, F., 1995. Apoptosis-related mortality in vitro of mononuclear cells from patients with HIV infection correlates with disease severity and progression. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. 9 (5), 450–458.

Peden, K., Emerman, M., Montagnier, L., 1991. Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1LAI, HIV-1MAL, and HIV-1ELI. Virology 185 (2), 661–672.

Penn, M.L., Grivel, J.C., Schramm, B., Goldsmith, M.A., Margolis, L., 1999. CXCR4 utilization is sufficient to trigger CD4+ T cell depletion in HIV-1-infected human lymphoid tissue. Proc. Natl. Acad. Sci. U.S.A. 96 (2), 663–668.

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 (4), 2855–2864.

Plymale, D.R., Tang, D.S., Comardelle, A.M., Fermin, C.D., Lewis, D.E., Garry, R.F., 1999. Both necrosis and apoptosis contribute to HIV-1-induced killing of CD4 cells. AIDS 13 (14), 1827–1839.

Renkema, G.H., Saksela, K., 2000. Interactions of HIV-1 NEF with cellular signal transducing proteins. Front. Biosci. 5, D268–D283.

Roshal, M., Zhu, Y., Planelles, V., 2001. Apoptosis in AIDS. Apoptosis 6 (1–2), 103–116.

Salvi, R., Garbuglia, A.R., Di Caro, A., Pulciani, S., Montella, F., Benedetto, A., 1998. Grossly defective nef gene sequences in a human immunodeficiency virus type 1-seropositive long-term nonprogressor. J. Virol. 72 (5), 3646–3657.

Schwartz, O., Marechal, V., Le Gall, S., Lemonnier, F., Heard, J.M., 1996. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat. Med. 2 (3), 338–342.

Simmons, A., Aluvihare, V., McMichael, A., 2001. Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. Immunity 14 (6), 763–777.

Spira, A.I., Ho, D.D., 1995. Effect of different donor cells on human immunodeficiency virus type 1 replication and selection in vitro. J. Virol. 69 (1), 422–429.

Stern, J.B., Smith, K.A., 1986. Interleukin-2 induction of T-cell G1 progression and c-myb expression. Science 233 (4760), 203–206.

Theodore, T.S., Englund, G., Buckler-White, A., Buckler, C.E., Martin, M.A., Peden, K.W., 1996. Construction and characterization of a stable full-length macrophage-tropic HIV type 1 molecular clone that directs the production of high titers of progeny virions. AIDS Res. Hum. Retroviruses 12 (3), 191–194.

Waldhuber, M.G., Bateson, M., Tan, J., Greenway, A.L., McPhee, D.A., 2003. Studies with GFP-Vpr fusion proteins: induction of apoptosis but ablation of cell-cycle arrest despite nuclear membrane or nuclear localization. Virology 313 (1), 91–104.

Willey, R.L., Smith, D.H., Lasky, L.A., Theodore, T.S., Earl, P.L., Moss, B., Capon, D.J., Martin, M.A., 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. 62 (1), 139–147.

Wu, L., Paxton, W.A., Kassam, N., Ruffing, N., Rottman, J.B., Sullivan, N., Choe, H., Sodroski, J., Newman, W., Koup, R.A., Mackay, C.R., 1997. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. J. Exp. Med. 185 (9), 1681–1691.

Zaunders, J.J., Moutouh-De Parseval, L., Kitada, S., Reed, J.C., Rought, S., Genini, D., Leoni, L., Kelleher, A., Cooper, D.A., Smith, D.E., Grey, P., Estaquier, J., Little, S., Richman, D.D., Corbeil, J., 2003. Polyclonal proliferation and apoptosis of CCR5+ T lymphocytes during primary human immunodeficiency virus type 1 infection: regulation by interleukin (IL)-2, IL-15, and Bcl-2. J. Infect. Dis. 187 (11), 1735–1747.

Zhang, Y.J., Fadeel, B., Hodara, V., Fenyo, E.M., 1997. Induction of apoptosis by primary HIV-1 isolates correlates with productive infection in peripheral blood mononuclear cells. AIDS 11 (10), 1219–1225.