CD4 downregulation by the human immunodeficiency virus type 1 Nef protein is dispensable for optimal output and functionality of viral particles in primary T cells

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INTRODUCTION

The regulatory Nef protein of human immunodeficiency virus types 1 and 2 (HIV-1/2) and simian immunodeficiency virus (SIV) is abundantly expressed early during infection and is essential in vivo for high levels of viral replication and disease progression (Deacon et al., 1995; Hanna et al., 1998; Kestler et al., 1991; Kirchhoff et al., 1995). Understanding the precise mechanisms through which Nef induces viral pathogenesis turned out to be difficult, mainly due to the large number of Nef biological activities which include: downregulation of cell surface molecules such as CD4 and MHC-I, alteration of signal transduction pathways, stimulation of virion infectivity and enhancement of viral replication (Malim & Emerman, 2008). A pathogenic role for Nef-induced CD4 downregulation was suggested by studies showing that viral strains expressing mutated Nef proteins, inactive on CD4, replicated at low levels and/or had a reduced/absent pathogenic potential in infected animal models (Hanna et al., 2006; Iafrate et al., 2000; Stoddart et al., 2003). Studies with animal models and HIV-1-infected individuals showed that nef alleles isolated after the development of immunodeficiency have a higher CD4 downregulation activity relative to those isolated during early asymptomatic stages of infection or derived from non-progressors (Carl et al., 2001; Casartelli et al., 2003; Mariani et al., 1996; Patel et al., 2002; Tobiume et al., 2002). However, it is currently unclear how Nef-mediated CD4 downregulation may increase in vivo viral loads and influence pathogenesis.

Nef acts as a CD4-specific sorting adaptor by accelerating receptor endocytosis and misdirecting internalized molecules to lysosomes where they are degraded instead of entering recycling pathways (Oldridge & Marsh, 1998). This process requires a membrane-proximal dileucine motif of CD4 (LL144) that functions as an internalization signal (Aiken et al., 1994), as well as various residues in Nef (WL58, MGxxxS, LL165 and DD175) (Geyer et al., 2001). In addition, a strong Nef-induced reduction of the half-life and anterograde transport of nascent CD4 molecules was demonstrated in transfected epithelial cell lines (Rose et al., 2005).
An important effect of Nef-mediated CD4 downregulation observed in 293T cells transfected with HIV-1 proviral DNA and CD4 expression vector consists in avoiding Env sequestration into a CD4–Env complex. As a result, progeny virions are released more efficiently and incorporate higher amounts of Env products, thus becoming more infectious (Lama et al., 1999; Ross et al., 1999). Nevertheless, it is well established that Nef can enhance the infectivity of virions in a manner that is independent of CD4 expression in producer cells and that involves early steps of the viral cycle in the target cell (Aiken & Trono, 1995; Chowers et al., 1995; Schwartz et al., 1995). In fact, some Nef mutants impaired in CD4 down-modulation were fully capable of enhancing virion infectivity (Glushakova et al., 2001; Goldsmith et al., 1995; Lundquist et al., 2002). Moreover, Nef can enhance the membrane expression and virion incorporation of Env products independently of CD4 downregulation (Schiavoni et al., 2004). By means of Nef mutants and patient-derived Nef proteins, a correlation between the efficiency of CD4 downregulation and the capacity to stimulate HIV-1 replication in primary T cells was found (Fackler et al., 2006; Glushakova et al., 2001; Lundquist et al., 2002). In these studies, however, the relative CD4 downregulation activity of Nef variants poorly correlated with their ability to enhance viral infectivity and the efficiency of Env incorporation into virions has not been investigated. A possible cause of controversy resides in the fact that Nef, by using diversified cell type-dependent mechanisms to decrease surface CD4 expression, may have an impact in the production and functionality of HIV-1 particles that varies between different cell systems. We showed previously that, in Nef-expressing T cell lines, CD4 downregulation results mainly from accelerated endocytosis rather than impaired transport of neo-synthesized receptor that is due to a separate Nef activity (Giolo et al., 2007). The opposite is true for epithelial cells in which Nef has a little and strong effect on CD4 endocytosis and transport, respectively, probably as a reflection of the constitutive receptor turnover that is much faster than in T cells (Giolo et al., 2007; Rose et al., 2005). Here, we have further investigated the impact of the CD4 downregulation activity of Nef in the outcome and functionality of HIV-1 particles in both 293T cells and primary CD4^+ T lymphocytes.

RESULTS

**Downregulation of surface CD4 and enhancement of Env incorporation into virions are two separate Nef activities**

It was shown that CD4 expression in HIV-transfected 293T cells resulted in the release of viral particles with reduced Env levels and infectious capacity and that such inhibition was counteracted by the Nef and, to a smaller extent, Vpu viral proteins through their CD4 downregulation activity (Lama et al., 1999). In this cell system Nef and Vpu should primarily affect the anterograde transport of neo-synthesized CD4 molecules, as shown in previous reports (Giolo et al., 2007; Rose et al., 2005; Willey et al., 1992). To confirm this assumption, we analysed by immunofluorescence microscopy the intracellular distribution of CD4 in 293T cells transfected with a CD4-encoding vector alone or together with HIV-1 proviral DNA either wild-type (wt), defective for the expression of Nef (ΔNef), Vpu (ΔVpu) or both viral proteins (ΔVpuΔNef). Upon expression of wt virus, the intracellular CD4-specific staining accumulated in large vesicular structures close to the nucleus (Fig. 1a). The formation of these positive vesicle aggregates was dependent on the presence of Nef, since it occurred also in cells expressing ΔVpu but not ΔNef or ΔVpuΔNef viruses. As measured by FACS analysis, the cell-surface CD4 expression was suppressed by wt virus by about fivefold (Fig. 1b). This activity was maintained in the absence of Vpu, but reduced when Nef alone or both Nef and Vpu were not expressed (three- and twofold inhibition, respectively). Apparently, the inhibitory effect of HIV-1 on surface CD4 expression is Vpu-independent and relies mainly, although not exclusively, on Nef. An additive effect is possibly due to the capacity of Env to bind CD4 molecules in the endoplasmic reticulum and prevent their transport to the cell membrane (Geleziunas et al., 1994). Moreover, Western blotting analysis showed that expression of wt or Vpu-defective viruses resulted in the decrease of cell-associated CD4 (Fig. 1c). The CD4 reduction ranged between 10 and 40 % and was observed in six independent experiments (P<0.001 by paired Student’s t-test). Conversely, the steady-state levels of CD4 were not reduced upon expression of ΔNef or ΔVpuΔNef viruses. The transfection efficiency of the various HIV-1 constructs employed was always comparable among transfectants based on p24 Gag capsid antigen expression (Fig. 1c). The results indicate that expression of CD4 is mainly affected by Nef through its capacity to induce intracellular retention and degradation of nascent CD4 molecules. Indeed, we found that the surface expression of CD4LLAA, a CD4 mutant unresponsive to Nef-mediated endocytosis due to alanine substitution of leucines 143–144 (Aiken et al., 1994), was suppressed more efficiently by wt (fivefold) than ΔNef virus (threefold), analogously to what observed with CD4 (Fig. 2a). In addition, wt but not ΔNef virus reduced the steady-state levels of CD4 and CD4LLAA (Fig. 2b). Therefore, during simultaneous expression of HIV-1 and CD4, Nef downregulates CD4 in a manner that is independent of receptor endocytosis. These data suggest that Nef capacity to contrast the negative effect of CD4 on Env incorporation into virions may be mechanistically unlinked to the protein activity of CD4 internalization. To test this hypothesis, we analysed the composition of wt and ΔNef viral particles produced by 293T cells without CD4 expression, expressing CD4 or CD4LLAA. The purified viruses were analysed by Western blotting using antibodies specific for gp120 and gp41 Env products and the incorporation of these proteins was quantified (Fig. 2c). When viruses were released in the absence of CD4, the levels of incorporated gp120 and gp41 were slightly reduced in ΔNef (87±7 %) if compared with wt virus. In viruses released by CD4-expressing cells, the levels of
gp120 and gp41 were reduced for wt (82 ± 6% gp120 and 64 ± 4% gp41) and, to a much higher extent, for ΔNef (20 ± 2% gp120 and 24 ± 16% gp41), according to previously reported data (Lama et al., 1999). Notably, CD4LLAA also inhibited Env incorporation into ΔNef than in wt virions more efficiently. This inhibition was stronger than that of CD4, probably due to higher levels of CD4LLAA in producer cells (Fig. 2b). Therefore, Nef counteracted the inhibitory effect of CD4 on virion composition independently of the receptor dileucine motif. CD4 and CD4LLAA accumulated into virions to extents proportional to their surface expression and thus ~1.5-fold higher in ΔNef virions. Along with reduced Env levels, the amount of associated CD4 or CD4LLAA correlated with the reduction of virion infectivity (Fig. 2d). Finally, p24 and Nef were incorporated into virions regardless of CD4 or CD4LLAA expression (Fig. 2c).

Fig. 1. Nef induces CD4 retention and degradation in transfected 293T cells. 293T cells were transfected with pSV-CD4 (1 µg) alone or together with proviral DNA (20 µg) wt, ΔNef, ΔVpu or ΔVpuΔNef, and analysed 48 h later. (a) Cells were stained with anti-CD4 and anti-p24 antibody and processed for immunofluorescence microscopy. Individual channels corresponding to CD4 (top) and p24 (bottom) of optical sections acquired by confocal microscopy are shown. The arrows indicate the intracellular CD4 accumulation. Bar, 10 µm. (b) Cells were analysed by flow cytometry for the cell-surface expression of CD4. The mean fluorescence intensity (MFI) values of the life cell population are reported. The unshaded histogram shows labelling of cells not transfected. (c) Total cell lysates were analysed by Western blotting using antibodies against p24, Nef, CD4 and α-tubulin. The CD4 levels were normalized to tubulin and expressed by setting the sample expressing only CD4 at a 100%.

Nef capacity to avoid CD4-mediated virion retention in HIV-transfected cells does not occur through surface CD4 downregulation

The removal of surface CD4 by Nef in HIV-transfected 293T cells is believed to allow the release of progeny virions that would otherwise be trapped by CD4 at the plasma membrane (Ross et al., 1999). In light of our findings on the CD4–Nef interaction in this experimental system, we re-examined the role of Nef on virion release. First, we noticed that CD4LLAA accumulated at much higher levels than CD4 in cells transfected with the same amount of expression vectors (Fig. 2b). Since viral release could be influenced by CD4 in a dose-dependent manner, 293T cells have been transfected with wt or ΔNef proviral DNA, with or without an amount of pSVCD4 or pSVCD4LLAA vector that resulted in the same steady-state expression of CD4 or CD4LLAA, respectively, as determined in a pilot experiment (Fig. 3a). After transfection (48 h), p24 was measured in cellular lysates and cell culture media by Western blotting and ELISA, respectively, and the efficiency of viral release was calculated. If cells lacked CD4, the mean amounts of intracellular and supernatant p24 did not differ significantly between ΔNef and wt virus (data not shown). We found that both CD4 and CD4LLAA consistently increased the intracellular accumulation of p24 with a stronger effect on ΔNef virus (Fig. 3b shows one representative experiment of three). Accordingly, expression of CD4 or CD4LLAA resulted in an inhibition of viral particle release that was lower for wt (by 17 ± 2 and 44 ± 8%, respectively) than for ΔNef virus (by 73 ± 12 and 82 ± 4%, respectively) (Fig. 3c). These differences were statistically significant (P<0.05). If compared with CD4, CD4LLAA exerted a slightly stronger inhibition on both wt and ΔNef virus, possibly reflecting its higher stability at the cell membrane (Aiken et al., 1994). Importantly, Nef expression rescued virion release to the same extent (~threefold increase) whether this was inhibited by wt or mutated CD4. Therefore, the capacity of Nef to prevent the retention of progeny virions induced by CD4 is not mediated by accelerated receptor internalization.

To confirm the above findings with a Nef protein that more closely resembles primary variants, we used an NL4-3...
virus in which the resident nef gene has been replaced with one of the SF2 strains (wt-SF2Nef virus). We found that, in the 293T cell system, SF2 Nef reduced CD4 expression and its deleterious consequences on HIV-1 release and infectivity in a manner that was independent of CD4 endocytosis and to an extent similar to that of NL4-3 Nef (Fig. 4).

**CD4 downregulation by Nef in HIV-infected CD4+ T lymphocytes**

The above results suggest that Nef effects on viral particles produced by HIV-transfected cells may not be critical in T cells in which CD4 is constitutively expressed at the cell membrane and Nef-mediated CD4 down-regulation occurs primarily by accelerated internalization (Giolo et al., 2007; Rose et al., 2005). Thus, we generated three NL4-3-based proviral clones (Q37, L78 and K177) expressing Nef proteins with in vivo selected single amino acid substitutions (L37Q, P78L and E177K, respectively) that abrogate the CD4 downregulation activity (Casartelli et al., 2006; Giolo et al., 2007). These viruses, together with DDAA that expresses a Nef mutant, NEFDD175AA, defective for CD4 downregulation (Lu et al., 1998; Mangasarian et al., 1999), wt, wt-SF2Nef and ΔNef viruses, were used to infect freshly isolated primary CD4+ T lymphocytes. After viral exposure, T cells were activated and analysed by flow cytometry at various time points for the intracellular accumulation of p24 and the cell-surface expression of CD4. HIV-infected cells became apparent 5 days post-infection (p.i.) in terms of detectable p24 expression within cells and in the cell culture medium. Later on, the p24-specific fluorescence, the number of p24+ cells, and the amount of extracellular p24 progressively increased until reaching a maximum after about 10 days, then all parameters started to decline in accordance with massive cell death (Fig. 5 and data not shown). At 5 days p.i., the surface CD4 density of cells infected with wt and wt-SF2Nef was reduced to 20%, while cells infected with ΔNef or Nef-mutated viruses maintained 50–80% of CD4 expression (Fig. 5), in agreement with previous studies showing that Nef is the major determinant of CD4 down-modulation during the early phases of viral infection (Chen et al., 1996; Wildum et al., 2006). Notably, at 10 days p.i., a fivefold reduction of surface CD4 was also achieved in cells infected with ΔNef or Nef-mutated viruses, suggesting that Nef activity on CD4 may be irrelevant when the virus is productively assembled and released by primary T lymphocytes.
incorporation was much less dramatic than that observed in 293T cells. In viruses expressing Nef proteins defective on CD4, the incorporation of Env products was similar to that of wt, with the exception of Q37 that showed slightly reduced levels of gp41 (88 ± 5% of wt). If compared with wt virus, the amount of virion-associated Nef was higher in Q37 (144 ± 12%) and lower in L78 (48 ± 9%) virus, reflecting the relative levels of protein expression in producer cells (data not shown). Finally, in the viruses analysed p24 was equally incorporated, while CD4 was undetectable (the signal was indistinguishable from background in virus-free control sample).

**Nef role in the release of HIV-1 virions from primary T cells**

Next, we investigated the role of Nef and its CD4 downregulation activity in the production of HIV-1 particles from primary T cells. To achieve identical infection efficiencies, CD4+ T lymphocytes were infected with equivalent amounts of wt, ΔNef, Q37 or K177 viruses pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) (Chazal et al., 2001). Then, infected cells were activated and analysed after 42 h to assess the single-round release of viral particles. Surprisingly, both ΔNef and Nef-mutated viruses were released with efficiencies similar to that of wt (Fig. 7). Therefore, Nef has no role in the release of progeny virions in these cell settings.

**Nef mutants defective in CD4 downregulation have different capacities to stimulate HIV-1 infectivity and replication**

Finally, we analysed the impact of HIV-1 spread in Nef CD4 downregulation activity. Freshly isolated CD4+ T cells were infected with wt, ΔNef, Q37, L78 and K177 viruses, activated and analysed after 5 days to evaluate the percentage of p24+ cells as soon as they appeared. The ΔNef virus was eightfold less infectious than wt, in agreement with the CD4-independent capacity of Nef to enhance HIV-1 infectivity (Aiken & Trono, 1995; Goldsmith et al., 1995; Miller et al., 1995) (Fig. 8a). This Nef activity was completely absent in Q37, partially reduced in L78 and maintained by K177. The viruses were also tested for their replication potential in long-term T cell cultures by measuring soluble p24 in the culture supernatant at various times over a 2 week period. By comparing wt and ΔNef viruses, a positive effect of Nef was consistently observed on both viral growth rate and maximal titres achieved (Fig. 8b, c). The Q37 virus was much less infectious than wt, with the exception of Q37 that showed slightly reduced levels of gp41 (88 ± 5% of wt). If compared with wt virus, the amount of virion-associated Nef was higher in Q37 (144 ± 12%) and lower in L78 (48 ± 9%) virus, reflecting the relative levels of protein expression in producer cells (data not shown). Finally, in the viruses analysed p24 was equally incorporated, while CD4 was undetectable (the signal was indistinguishable from background in virus-free control sample).

**Regulation of virion composition by Nef variants defective for CD4 downregulation**

The structural composition of wt, ΔNef and Nef-mutated viruses released by infected primary CD4+ T lymphocytes was analysed. To achieve high viral titres and bypass the requirement for a functional Nef protein to reach optimal HIV-1 replication (Spina et al., 1994), CD4+ T cells were activated and then, 3 days later, were infected. When 40–60% of the cells were undergoing productive infection (6 days p.i.), the viruses were purified from culture supernatant and analysed by Western blotting (Fig. 6). If compared with wt, ΔNef had consistently reduced levels of gp120 (80 ± 13%), while only one out of three viral preparations displayed lower gp41 levels (82%, shown in Fig. 6). Therefore, in T cells the effect of Nef on Env
DISCUSSION

Our data indicate that the previously described Nef capacity to prevent the negative effects of CD4 on the composition and release of HIV-1 particles in 293T cells (Lama et al., 1999; Ross et al., 1999) resulted from retention/degradation rather than accelerated endocytosis of the receptor. In fact we showed that, in this cell system, the deleterious consequences for the virus of CD4 expression were contrasted by Nef even though the receptor was unresponsive to Nef-mediated endocytosis (i.e. CD4LLAA). These results are at odd with previous studies showing that Nef does not rescue the inhibition on virion release caused by CD4LLAA (Ross et al., 1999). In this regard, we observed that CD4LLAA accumulates at much higher levels than CD4 and interferes with HIV-1 release in a dose-dependent manner. By measuring the efficiency of virion release from cells expressing equivalent levels of CD4 or CD4LLAA, we have been able to show that Nef relieves the inhibition of both molecules and thus operates in a manner that is independent from its ability to downregulate surface CD4.

In contrast with a previous report (Tanaka et al., 2003), we could not measure the contribution of Vpu to CD4 degradation in transfected 293T cells. Divergent observations could be due to differences in basal CD4 expression levels that may have saturated the Vpu-mediated mechanism of CD4 degradation in our experimental settings. Recent reports showed that Vpu antagonizes tetherin, a viral restriction factor that limits HIV-1 release and is not expressed by 293T cells (Neil et al., 2008; Van Damme et al., 2008). Therefore, the impact on progeny virions of the CD4 downregulation activity of Vpu warrants future investigation in natural HIV-1 cell targets by means of

Fig. 4. SF2 Nef contrasts the negative effects of CD4 like NL4-3 Nef in a manner that is independent of receptor endocytosis. (a, b) 293T cells transfected with the indicated vectors were analysed as described in Figs 1(b) and 3(b). (c, d) The release and the infectivity of wt-SF2Nef and ΔNef viruses produced by 293T cells with or without expression of CD4 or CD4LLAA were evaluated as described in Figs 3(c) and 2(d), respectively. Three independent experiments have been performed and either one set of representative results (a, b) or mean values ± SD are shown (c, d).

Fig. 5. Levels of surface CD4 in primary CD4+ T cells infected with HIV-1 wt, Nef-deficient or expressing Nef mutants defective for CD4 downregulation. Freshly isolated CD4+ T lymphocytes were not infected (n.i.) or infected with the indicated viruses, then activated. At various times p.i., expression of cell-surface CD4 and intracellular p24 was analysed by two-colour flow cytometry. (a) MFI values specific for CD4 of n.i. cells and of gated p24+ cells are shown at 5 and 10 days p.i. Some CD4+ p24+ cells appeared at 10 days p.i., probably due to the binding of soluble gp120 and progeny virions to CD4 on the surface of uninfected T cells that caused receptor down-modulation or masking. (b) The mean ± SD intensity of fluorescence relative to CD4 was determined in three independent experiments. Data were calculated by considering 100% the CD4 expression of n.i. cells. Significant differences between wt and other viruses are indicated: *, P < 0.05; **, P < 0.01.
yet unidentified Vpu mutants that have lost this activity and maintain or not the capacity to antagonize tetherin.

Data presented here show that the role of Nef on the expression and antiviral functions of CD4 in infected primary CD4+ T lymphocytes differed in various ways compared to transfected 293T cells. Shortly after HIV-1-infected of CD4+ T cells, Nef is essential for the removal of CD4 molecules already expressed on the cell surface while later on, when T cells are productively assembling and releasing viral particles, maximal CD4 downregulation is achieved in a Nef-independent manner, probably through the activities of the late viral proteins Vpu and Env (Geleziunas et al., 1994). Moreover, we showed that in infected CD4+ T lymphocytes Nef had a modest effect on the incorporation of gp120 and gp41 independently of its activity on CD4 and did not influence the overall release of progeny virions.

The CD4 downregulation is a function of Nef highly conserved in multiple HIV-1 strains, HIV-2 and SIV (Schindler et al., 2006), thus it must be important for lentiviral replication. Our data support a role for this Nef activity in the early rather than late phases of the HIV-1 life cycle. Possibly, Nef-mediated CD4 downregulation is required to counteract events occurring soon after infection that would otherwise lead to inefficient viral replication. Indeed, the ability to reduce surface CD4 contributes at least in part to Nef capacity to prevent viral superinfection (Benson et al., 1993; Wildum et al., 2006), a phenomenon that can cause premature cell death. In addition, rapid CD4 downregulation by Nef may protect HIV-infected cells by preventing proapoptotic signals that are delivered through the receptor by Env (Ahr et al., 2004). This latter model implies a crucial role for Nef activity on CD4 in the spread of
HIV-1 at the sites of infection and deserves to be investigated in future studies.

We found that the CD4 downregulation activity of Nef poorly correlated with the protein capacity to stimulate viral infectivity and replication in primary CD4+ T lymphocytes. These results are in line with previous reports showing that Nef mutants impaired in CD4 down-modulation maintained the capacity to enhance infectivity (Glushakova et al., 2001; Goldsmith et al., 1995; Lundquist et al., 2002) and to increase the membrane expression of gp41 and gp120 (Schiavoni et al., 2004). On the other hand, some studies reported Nef mutants defective in CD4 downregulation that had lost the capacity to enhance Env incorporation into virions and/or to stimulate viral replication in primary T cells (Fackler et al., 2006; Glushakova et al., 2001; Lundquist et al., 2004). This discrepancy may be explained by the fact that separate functions of Nef can be mediated by the same protein surface, as in the case of Leu37 that is required for the capacity to decrease surface CD4 expression as well as for Nef independent activities on HIV-1 infectivity and replication.

In summary, our findings bring novel insights into the role of Nef in the viral life cycle that may possibly help the identification of effective anti-HIV therapeutic strategies.

METHODS

Cells and antibodies. 293T and CEM-GFP (Gervaix et al., 1997) cells were maintained in Dulbecco’s modified Eagle’s and RPMI 1640 medium, respectively, supplemented with 10% FBS, 2 mM L-glutamine, 100 U penicillin–streptomycin ml−1 (all from Gibco). For CEM-GFP, the medium was also supplemented with 100 µg G418 (Gibco) ml−1. The antibodies used for flow cytometry, immunofluorescence microscopy and Western blotting analysis of CD4, Nef and p24 were as described previously (Casartelli et al., 2003; Cerboni et al., 2003; Doria et al., 2007). Western blot analysis was also performed with anti-gp160/gp120 goat serum (HT3), anti-p24 rabbit serum (CA1), anti-tubulin (Sigma) or anti-gp41 (Chessie 8, anti-TM) mouse monoclonal, and human serum from an HIV-1-infected individual.

DNA constructs. Proviral HIV-1 constructs were generated in the pNLblue Nco nefNot vector (Fackler et al., 2006). The mutated NL4-3 nef gene was amplified by PCR with specific primers introducing Ncol and NotI sites at the 5’ and 3’ ends, respectively, and cloned in the Ncol/NotI sites replacing the wt nef gene. DNA constructs were confirmed by sequencing of both strands.

HIV-1 production, purification and detection. Stocks of NL4-3 viruses either wt, expressing SF2 Nef (wt-SF2Nef or WT/NL4-3 SF2 Nef described in Fackler et al., 2006), expressing Nef mutated by single amino acid substitutions (Q37, L78 and K177), or unable to express Nef (ΔNef) (Chowers et al., 1994), Vpu, or both Nef and Vpu (pBRNL43vpu ΔVpu and pBRNL43vpu nef ΔVpuNef HIV-1 clones, respectively, kindly provided by Frank Kirchhoff, University of Ulm, Germany) were prepared by transfecting with the standard calcium-phosphate method 20 µg proviral plasmids into 293T cells. In some experiments, cells were also transfected with 3.5 µg of a VSV-G-expressing plasmid (pCMV-VSV-G) or with indicated amounts of a vector encoding CD4 either wt or mutated at the dileucine motif (413–414), thus unresponsive to Nef-induced downregulation (pSVC4 and pSVC4LLAA, respectively; Gratton et al., 1996). Post-transfection (48 h), cell culture supernatants were collected, clarified by low-speed centrifugation, and stored in aliquots at −80°C. For the analysis of virion composition, viruses had been purified by ultracentrifugation through a sucrose cushion as described previously (Schiavoni et al., 2004). Viral stocks were titrated by reverse transcriptase assay (data not shown) and/or anti-p24 ELISA (Immunogenetics) according to the manufacturer’s instructions. For each experiment, at least three different stocks of viruses were used.

HIV-1 infection. To evaluate viral infectivity, CEM-GFP indicator cells were used as described elsewhere (Doria et al., 2009). For HIV-1 infection of primary human CD4+ T lymphocytes, purified cells were derived from healthy donors as described previously (Cerboni et al., 2007). To analyse HIV-1 expression in infected cells and evaluate the efficiency of single-cycle infection, CD4+ T cells were infected by incubation for 4 h at 37°C with 50 ng p24 per 106 cells of virus (corresponding to an m.o.i. of 0.003). Cells were then washed twice, resuspended at 1.5×106 ml−1 in complete RPMI 1640 medium supplemented with 100 IU human rIL-2 ml−1, and stimulated by the addition of 3 µg PHA (Sigma) ml−1 and irradiated allogeneic peripheral blood mononuclear cells at a 1:1 ratio. Finally, infected CD4+ T cells were harvested after 3, 5, 7 and 10 days for FACS analysis. The efficiency of viral infectivity was calculated as percentage of p24+ cells 5 days p.i. setting the value obtained with wt virus at 100%. To analyse HIV-1 replication, CD4+ T cells were infected and activated as described above, but using 12.5 ng p24 per 106 cells. In addition, over a 2 week period, an aliquot of cell culture supernatant was collected every 2–3 days for p24 quantification and replaced with fresh medium. To obtain high viral titres for virion purification, CD4+ T cells were first activated as described above and then, after 3 days, infected with 50 ng p24 per 106 cells of virus. After 6 days, cell culture supernatant was collected to purify virus by ultracentrifugation and cells were harvested for FACS and Western blot analysis.

Immunofluorescence microscopy. 293T cells were grown on glass coverslips and transfected with the indicated DNA. After 48 h, cells were fixed, permeabilized and processed for two colour immunofluorescence as described elsewhere (Gioio et al., 2007). The confocal imaging was performed on Olympus Fluoview FV1000 confocal IX81 inverted microscope equipped with FV10-ASW version 1.6 software.

Flow cytometry. For simultaneous detection of surface CD4 and intracellular p24 in HIV-1-infected CD4+ T cells, 5×105 infected or uninfected cells were incubated with anti-CD4 mAb or mouse IgG1 as isotype control. After three washes, cells were incubated with Cy5-conjugated goat anti-mouse IgG. Cells were then washed, fixed and permeabilized with reagents from BD Biosciences, incubated with normal mouse serum and then with PE-conjugated anti-p24 mAb. Finally, cells were washed, resuspended in 1% paraformaldehyde and analysed by two colour flow cytometry on a FACSCalibur with CellQuest software (BD Biosciences). To detect surface CD4 in 293T cells, 48 h post-transfection cells were stained with PE-conjugated anti-CD4 Ab, then treated and analysed as described above.

Western blot analysis. Both cells and purified viruses were lysed in buffer containing 1% Triton X-100 as described elsewhere (Casartelli et al., 2003). Equal amounts of total cellular lysates (20 µg) or viral preparations (corresponding to 2 µg p24 by ELISA) were separated by 10% SDS-PAGE and analysed by immunoblotting with appropriate antibodies and the ECL system (Amersham Pharmacia Biotech) as described previously (Casartelli et al., 2003). Protein-specific signals were quantified by densitometry.

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