HIV-1 infection generates extrachromosomal circular forms of viral DNA (E-DNA) in addition to the linear DNA product, which serves as the precursor to integrated proviral DNA (Pang et al., 1990; Pauza & Galindo, 1989; Shaw et al., 1984). Circular E-DNA, formed following host-mediated circularization of the linear genome, includes 1-long terminal repeat circles (1-LTR) and 2-LTR circles (Farnet & Haseltine, 1991; Li et al., 2001; Pauza et al., 1989). While HIV-1 replication is known to generate these forms of E-DNA, their significance in virus replication and pathogenesis is poorly understood. Although unable to support productive replication, HIV-1 E-DNA does not support productive replication, it is transcriptionally active and produces viral proteins. However, the significance of E-DNA in virus replication and pathogenesis is poorly understood. In this study, the functional activity of the HIV-1 Nef protein expressed in the absence of viral integration was analysed. Using both a recombinant HIV-1 IN defective virus and a diketo acid IN inhibitor, evidence was provided showing that Nef expressed from E-DNA downregulates CD4 surface expression on primary CD4+ T lymphocytes. These results suggest that proteins expressed in the absence of integration may have potential clinical consequences, an issue that should be further explored with the introduction of IN inhibitors.

Recently developed integrase inhibitors targeting the HIV-1 integrase (IN) protein block integration of HIV DNA in the target cell, preventing subsequent virus replication. In the absence of integration, viral DNA is shunted towards the formation of extrachromosomal DNA (E-DNA). Although HIV-1 E-DNA does not support productive replication, it is transcriptionally active and produces viral proteins. However, the significance of E-DNA in virus replication and pathogenesis is poorly understood. In this study, the functional activity of the HIV-1 Nef protein expressed in the absence of viral integration was analysed. Using both a recombinant HIV-1 IN defective virus and a diketo acid IN inhibitor, evidence was provided showing that Nef expressed from E-DNA downregulates CD4 surface expression on primary CD4+ T lymphocytes. These results suggest that proteins expressed in the absence of integration may have potential clinical consequences, an issue that should be further explored with the introduction of IN inhibitors.

In this study, both recombinant IN defective virus and an IN inhibitor were used to evaluate the ability of the viral accessory protein Nef, expressed in the absence of viral integration, to mediate CD4 downregulation in activated primary CD4+ T lymphocytes. HIV-1 Nef has been implicated in the downregulation of CD4 in both CD4+ expressing cell lines, and in Nef overexpression systems (Aiken et al., 1994; Lundquist et al., 2002; Mangasarian et al., 1997; Rhee & Marsh, 1994). However, only recently has this observation been extended to include the primary CD4+ T lymphocyte in the context of HIV-1 infection (Lundquist et al., 2002). To verify that Nef-mediated downregulation was detectable in primary CD4+ T lymphocytes, lymphocytes were infected with recombinant HIV-1 competent or defective for Nef expression (IN+/Nef+ and IN+/Nef−, respectively). Vesicular stomatitis virus (VSV)-pseudotyped, replication-defective viruses were produced in HEK-293T...
cells using a three-plasmid co-transfection system described by Naldini et al. (1996). Viral supernatants were normalized based on p24 (SAIC-Frederick). The following plasmids were utilized for virus production: pMD.G (D. Trono, Salk Institute, LaJolla, CA) provided the VSV.G glycoprotein and pCMVARE8.2 (I. Verma, Salk Institute, LaJolla, CA) served as the packaging construct; pNL4-3:ΔG/P-EGFP (M. D. Ross, Mount Sinai, New York, USA) and pNL4-3:ΔG/P-EGFP/DNef (M. Husain, Mount Sinai, New York, USA) clones, derived from HIV-1NL4-3, were packaged within the virions (Husain et al, 2002). Both packaged constructs contained a deletion within gag-pol into which enhanced green fluorescence protein (EGFP) was cloned, as well as a point mutation in env. The deletion and point mutation rendered the virus replication defective. The plasmid pNL4-3:ΔG/P-EGFP/DNef contained a mutation within the nef ATG site and did not produce Nef protein as demonstrated previously (Husain et al, 2002).

Primary CD4+ T lymphocytes isolated from the peripheral blood mononuclear cells of normal blood donors using the MACS CD4+ T cell isolation kit (Miltenyi Biotec) were activated with 5 μg PHA (Sigma-Aldrich) ml-1. The CD4+ T lymphocyte population was 98% pure as determined by staining with antibodies against CD3 and CD4 (BD Pharmingen) (data not shown). Activated CD4+ T lymphocytes (5 × 10^5) were incubated with 200 ng p24 of IN+/Nef+ or IN+/Nef– virus at 37°C overnight. At 72 h post-inoculation the lymphocytes were analysed. CD4 expression levels were measured on the infected population, as determined by EGFP expression, using anti-CD4 antibody conjugated to allophycocyanin (BD Pharmingen) (Fig. 1a). Cells were analysed on a FACS Calibur flow cytometer (Becton Dickinson) and data were analysed using FlowJo software (Tree Star). Eighty-eight percent of mock-infected cells and 86% of cells infected with control virus (CMV-EGFP), encoding the viral LTRs in the absence of other viral proteins, were positive for CD4 expression (Fig. 1b). While the number of CD4+ T cells decreased to 50% in the IN+/Nef– virus-infected population, expression levels decreased more significantly to 6% in the presence of Nef, as observed in the cell population infected with IN+/Nef+. The decrease in CD4 expression levels observed in the absence of Nef can be attributed to Vpu, which also has CD4 downregulating activity (Chen et al, 1996). These findings are consistent with previous reports demonstrating that CD4 expressed on primary CD4+ T lymphocytes is downregulated by HIV-1 Nef (Lundquist et al, 2002).

Fig. 1. Nef-mediated CD4 downregulation in primary CD4+ T lymphocytes. CD4 expression levels were determined by flow cytometry on activated primary CD4+ T lymphocytes inoculated with control virus (CMV-EGFP), or virus defective or competent for Nef expression (IN+/Nef– or IN+/Nef+, respectively). Levels were measured on the infected cell populations as determined by EGFP expression. A total of 10 000 events were counted. (a) Flow cytometry results. (b) Graphical depiction of the percentage CD4+ T lymphocytes. P-values were calculated using one-way analysis of variance (ANOVA). NS, Not significant. Results are representative of three experiments each performed in duplicate.

IN-defective HIV-1 (IN–) has proven to be a useful tool for studying HIV-1 E-DNA (Ansari-Lari et al, 1995; Cara et al, 1996, 2002; Engelman et al, 1995; Fang et al, 2001; Nakajima et al, 2001; Poon et al, 2003; Stevenson et al, 1990a, b; Teo et al, 1997; Wiskerchen et al, 1995; Wu et al, 2001, 2003). A point mutation in the IN coding region results in IN protein defect in strand transfer activity, subsequently shunting viral DNA towards the formation of E-DNA in the absence of integration (Engelman et al, 1995; Wiskerchen et al, 1995). Using recombinant IN– HIV-1, we evaluated the ability of Nef expressed from E-DNA to downregulate CD4. IN– virus was generated using the packaging construct pHelpIN+ (J. Reiser, Louisiana State UHSC, New Orleans, LA), which contained a point mutation (D116N) within the IN coding region of pol (Mochizuki et al, 1998).

Consistent with previous studies performed in cell lines, infection of activated primary CD4+ T lymphocytes with 200 ng p24 equivalent IN– virus resulted in increased ratios
of 2-LTR circles (E-DNA) to total HIV DNA compared with infection with IN + virus (data not shown) (Engelman et al., 1995; Wiskerchen et al., 1995). Viral protein expression was evaluated in lymphocytes infected with IN− virus by measuring virus-encoded EGFP. EGFP expression in mock-infected lymphocytes was subtracted as background. Consistent with previous findings, CD4+ T lymphocytes infected with IN− virus had detectable levels of EGFP, albeit a threefold lower mean fluorescent intensity of EGFP than seen in IN+ virus-infected lymphocytes (33 and 105, respectively) (Engelman et al., 1995; Wiskerchen et al., 1995). Viral protein expression was evaluated in lymphocytes infected with IN+ virus by measuring virus-encoded EGFP. EGFP expression in mock-infected lymphocytes was subtracted as background. Consistent with previous findings, CD4+ T lymphocytes infected with IN+ virus had detectable levels of EGFP, albeit a threefold lower mean fluorescent intensity of EGFP than seen in IN+ virus-infected lymphocytes (33 and 105, respectively) (Fig. 2a) (Ansari-Lari et al., 1995; Cara et al., 1996; Engelman et al., 1995; Nakajima et al., 2001; Wiskerchen et al., 1995).

To verify that Nef was transcribed from HIV-1 E-DNA in our system, RNA was isolated from CD4+ T lymphocytes infected with IN+ or IN− virus, and analysed for Nef-encoding mRNA. Nef-specific message was amplified from total RNA by one-step RT-PCR (Qiagen) using primers and cycling parameters described previously (Klotman et al., 1991). Amplification products were visualized by ethidium bromide staining following electrophoresis through 4–20% TBE polyacrylamide gels (Bio-Rad). Identification of Nef-encoding mRNA was confirmed by hybridization with a probe specific for multiply spliced HIV-1 mRNA (data not shown). Amplification of Nef-specific mRNA was not detected in mock-infected cells but was detected in both IN+ and IN− virus-infected cells at 27 and 53 h post-infection (Fig. 2b). These data, consistent with previous studies, demonstrated that Nef was transcribed from HIV-1 E-DNA in primary CD4+ T lymphocytes (Hazuda et al., 2000; Wu et al., 2001, 2003).

To determine if Nef expressed in the absence of viral integration was functional, we evaluated Nef-mediated CD4 downregulation in primary CD4+ T lymphocytes infected with IN− virus either defective or competent for Nef expression (IN−/Nef− and IN−/Nef+, respectively). CD4 levels were analysed on the EGFP-positive lymphocytes, representing the fraction of infected lymphocytes (Fig. 2c).

**Fig. 2.** Nef-mediated CD4 downregulation following infection with IN defective virus. (a) Mean fluorescence intensity of virus-encoded EGFP expression levels in activated primary CD4+ T lymphocytes infected with IN-competent virus (IN+) or IN-defective (IN−) virus at 72 h post-infection were determined by flow cytometry. (b) Ethidium bromide-stained gel demonstrating amplification by RT-PCR of Nef-encoding mRNA in total RNA from infected T lymphocytes at 27 and 53 h post-infection. Mock-inoculated cells are included (M). (c) Flow cytometry for surface CD4 expression on CD4+ T lymphocytes mock-inoculated, or infected with IN− virus defective and competent for Nef expression (IN−/Nef− and IN−/Nef+, respectively). Levels were measured on the infected cell populations as determined by EGFP expression. Graphical depiction of the percentage CD4+ lymphocytes (right panel). Lymphocytes infected with control virus are labelled (CMV-EGFP). Flow cytometry results are based on 10,000 events. *P*-values were calculated using one-way analysis of variance (ANOVA). NS, Not significant. Results are representative of three experiments each performed in duplicate.
Eighty-eight percent of the mock-inoculated lymphocytes were positive for CD4, and this number did not change significantly in cells inoculated with control virus (82%) (Fig. 2c). Lymphocytes infected with IN−/Nef− virus had CD4 levels comparable to cells infected with control virus (80%) (Fig. 2c). However, infection with IN− virus expressing Nef (IN−/Nef+) resulted in a significant decrease in the percentage of CD4+ cells to 22%. The presence or absence of Nef did not alter EGFP expression or the ratio of 2-LTR circles to total HIV DNA (data not shown). In contrast to infection with IN+/Nef− virus, in which CD4 was downregulated in the absence of Nef, IN−/Nef+ virus infection of CD4+ T lymphocytes did not lead to significant decreases in CD4 levels. Although the IN− virus should encode Vpu, which also has CD4 downregulating activity, the low level of protein expressed from E-DNA, coupled with the observation by Chen et al. (1996) that Nef accounts for more of the CD4 downregulation observed in primary CD4+ T lymphocytes than Vpu, suggests that the levels of Vpu expressed may not be sufficient to downregulate CD4 (Chen et al., 1996).

While IN− virus can serve as a model for studying HIV-1 E-DNA, the kinetics of HIV infection in the presence of an IN inhibitor may be more clinically relevant than those observed with IN− virus. Wu et al. (2003) recently demonstrated that HIV-1 Nef is expressed in the presence of a diketo acid IN inhibitor (Wu et al., 2003). We evaluated the ability of Nef expressed in the context of IN+ virus infection in the presence of IN inhibitor to downregulate CD4 on primary CD4+ T lymphocytes. L731,988, a diketo acid kindly provided by Merck Research Laboratories, prevents viral integration by blocking strand transfer, the final catalytic step in integration (Hazuda et al., 2000; Vandegraaff et al., 2001). Activated primary CD4+ T lymphocytes were incubated with L731,988 at the previously determined inhibitory concentration of 10 μM, for 1 h at 37 °C prior to infection (Hazuda et al., 2000). Following infection with 200 ng p24 equivalent IN+/Nef− or IN+/Nef+ virus, the lymphocytes were maintained in the presence of inhibitor for the duration of the experiment.

The impact of L731,988 on the kinetics of infection was first evaluated by quantification of 2-LTR circles and late reverse transcription products by real-time PCR using primers, probes and cycling parameters described previously (Butler et al., 2001). Samples were analysed on the ABI Prism 7700 sequence detection system (PE-Applied Biosystems). Serial dilutions of pTA2LTR, a plasmid containing the full-length 2-LTR junction sequence, and the transfer vector pNL4-3:ΔG/P-EGFP, served as standard curves from which 2-LTR circles and total HIV DNA were quantified, respectively (Cara et al., 1996). Amplification of glyceraldehyde 3-phosphate dehydrogenase was performed to control for DNA concentration (Clontech). The ratio of E-DNA to total HIV DNA was determined using 2-LTR circles as representative of E-DNA. The ratio of 2-LTR circles to total HIV DNA increased four- to fivefold in the presence of the IN inhibitor to levels similar to those seen with IN− virus (0.18 and 0.13, respectively) (Fig. 3a). These results indicate that the virus is shunted towards the formation of E-DNA in the absence of integration, consistent with the work of Hazuda et al. (2000).

Expression levels of virus-encoded EGFP in the presence and absence of the IN inhibitor were also evaluated (Fig. 3b). EGFP levels decreased approximately twofold from 105 to 46 in the presence of L731,988, to levels indistinguishable from those observed in cells infected with IN− virus (Fig. 3b). These data strongly suggest that L731,988 prevents integration of the recombinant virus, generating transcriptionally and translationally active E-DNA. In the presence of L731,988, IN+ virus infection results in expression levels and DNA ratios indistinguishable from infection with IN− virus.

CD4 expression was evaluated on cells infected with IN+/Nef− or IN+/Nef+ virus in the presence and absence of the IN inhibitor (Fig. 3c). Ninety-five percent of the mock-inoculated T lymphocyte population was CD4+, and this percentage did not change significantly in lymphocytes infected with control virus (93%) (Fig. 3c). Additionally, CD4 levels on lymphocytes incubated with L731,988 alone were similar to those in the mock-inoculated lymphocyte population. Infection of CD4+ T lymphocytes with Nef expressing virus in the presence of IN inhibitor resulted in a significant decrease in the percentage of CD4+ T lymphocytes (67 to 39%), while infection with Nef− virus did not (Fig. 3c). It is important to note that the extent of Nef-mediated downregulation observed in cells infected with IN+ virus in the presence of IN inhibitor, was indistinguishable from downregulation observed in cells infected with IN− virus.

This study was designed to analyse the activity of the Nef protein expressed from HIV-1 E-DNA. Nef-mediated downregulation of CD4 was observed in lymphocytes infected with IN+ virus, consistent with previous reports (Lundquist et al., 2002). However, using both an IN− virus (D116N) and the IN inhibitor L731,988, we have demonstrated that Nef expressed in the absence of viral integration also downregulates surface CD4 on primary CD4+ T lymphocytes. Previous studies demonstrated that HIV-1 E-DNA expresses viral transcripts and proteins; however, this is the first demonstration that a protein expressed in the absence of integration can alter the phenotype of infected cells.

With the introduction of IN inhibitors as anti-retroviral therapy, it is important to understand the activity of virus in their presence. Here, we demonstrate that IN− virus provides an accurate tool with which to study virus in the context of IN inhibitors.

Expression of HIV-1 proteins from E-DNA may have implications for virus replication and pathogenesis. Recently it has been shown that 2-LTR circles, previously thought
to be short-lived, persist in growth-arrested T cell lines beyond their previously determined half-life (Butler et al., 2002; Pierson et al., 2002). The persistence of 2-LTR circles has also been observed in primary human macrophages, a natural target of HIV-1 infection in vivo (Gillim-Ross et al., 2005). Additionally, Brussel et al. (2003) recently demonstrated that anti-retroviral therapy has a low impact on 2-LTR circle levels in viva, despite a dramatic decrease in plasma viremia and infectious cell frequency (Brussel et al., 2003). The introduction of IN inhibitors to anti-retroviral therapy regimens increases our need to understand HIV-1 E-DNA.

Our data strongly suggest that not only are viral proteins expressed in the absence of integration, but they are also expressed at functional levels. In this study, we focused on the CD4 downregulating activity of HIV-1 Nef. However, several other activities have been attributed to Nef. Nef has been implicated in downregulation of surface major histocompatibility complex class I (MHC-I), enhancement of viral infectivity and modulation of T cell signalling pathways (reviewed by Cullen, 1998; Schwartz et al., 1996; Swingler et al., 1999, 2003). By downregulating the target cell's MHC-I, required for presentation of viral peptide epitopes to cytotoxic T lymphocytes (CTL), cells harbouring transcriptionally active E-DNA may be able to escape CTL-mediated cell lysis (Cullen, 1998). Nef expression has also been shown to induce CC-chemokine production and to induce chemotaxis and activation in adjacent resting lymphocytes, a role suggested to facilitate recruitment of susceptible, activated cells (Swingler et al., 1999, 2003). Conversely, persistent low-level expression of viral proteins

![Fig. 3. Nef expressed in the presence of IN inhibitor downregulates CD4. Activated primary CD4$^+$ T lymphocytes infected with IN competent virus in the absence (IN+) and presence (IN+/L731,988), of IN inhibitor or IN defective virus in the absence (IN-) and presence (IN-/L731,988) of IN inhibitor were collected at 72 h post-infection. (a) Ratio of 2-LTR circles to total HIV DNA was determined by real-time PCR. (b) Mean fluorescence intensity of virus-encoded EGFP expression levels in infected T lymphocytes was determined by flow cytometry. (c) Surface CD4 expression levels on primary T lymphocytes mock-inoculated or infected with IN+/Nef- or IN-/Nef+ virus in the absence or presence of IN inhibitor (IN+/Nef-/L731,988 and IN-/Nef+/L731,988, respectively) or with IN virus competent or defective for Nef expression (IN-/Nef+ and IN-/Nef-, respectively). Levels were measured by flow cytometry on the infected cell populations as determined by EGFP expression. Graphical depiction of the percentage CD4$^+$ lymphocytes (right panel). Mock inoculated (M) lymphocytes infected with control virus (CMV-EGFP), and lymphocytes incubated with L731,988 alone are included. Flow cytometry results are based on 10,000 events. P-values were calculated using one-way analysis of variance (ANOVA). NS, Not significant. Results are representative of three experiments each performed in duplicate.](http://vir.sgmjournals.org)
may lead to immune stimulation. While IN inhibitors would be used in the setting of multi-drug therapy, and the number of cells infected at any given time would probably be small, the transcriptional activity of E-DNA, coupled with its persistence in non-dividing cell populations, may have consequences for infected cells.

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