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CD4 and MHC class I down-modulation activities of *nef* alleles from brain- and lymphoid tissue-derived primary HIV-1 isolates

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Abstract

HIV-1 *nef* undergoes adaptive evolution in the CNS, reflecting altered requirements for HIV-1 replication in macrophages/microglia and brain-specific immune selection pressures. The role of Nef in HIV-1 neurotropism and the pathogenesis of HIV-associated dementia (HAD) is unclear. In this study, we characterized 82 *nef* alleles cloned from brain, CSF, spinal cord and blood/lymphoid tissue-derived HIV-1 isolates from 7 subjects with HAD. CNS isolate-derived *nef* alleles were genetically compartmentalized and had reduced sequence diversity compared to those from lymphoid tissue isolates. Defective *nef* alleles predominated in a brain-derived isolate from one of the 7 subjects (MACS2-br). The ability of Nef to down-modulate CD4 and MHC class I (MHC-I) was generally conserved among *nef* alleles from both CNS and lymphoid tissues. However, the potency of CD4 and MHC-I down-modulation was variable, which was associated with sequence alterations known to influence these Nef functions. These results suggest that CD4 and MHC-I down-modulation are highly conserved functions among *nef* alleles from CNS- and lymphoid tissue-derived HIV-1 isolates that may contribute to viral replication and escape from immune surveillance in the CNS.

Keywords

HIV-1; dementia; Nef; neurotropism; CNS; lymphoid; CD4; MHC-I

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Introduction

Human immunodeficiency virus type 1 (HIV-1) productively infects perivascular macrophages and microglia in the central nervous system (CNS) and causes HIV-associated dementia (HAD) and related neurological disorders in 10–20% of AIDS patients (Gonzalez-Scarano and Martin-Garcia, 2005). Non-productive infection of astrocytes is also prominent in HAD (Churchill *et al*, 2006b; Churchill *et al*, 2009). The HIV-1 accessory protein Nef has been implicated to have a role in the development of HAD by enhancing viral replication and viral loads in the CNS, production of proinflammatory cytokines/chemokines, and/or neuronal cell death, in addition to other unknown mechanisms (Thompson *et al*, 2003; van Marle *et al*, 2004). Nef, a 27 kDa membrane-associated cytoplasmic protein, has several key functions that include downmodulation of CD4 and MHC class 1 (MHC-1) molecules from the cell surface, enhancement of viral replication and infectivity, and modulation of cellular signalling pathways (Collins *et al*, 1998; Craig *et al*, 1998; Geleziunas *et al*, 2001; Lama *et al*, 1999). However, the requirement for Nef function is cell type dependent and can differ depending on the mode of viral entry (Aiken, 1997; Luo *et al*, 1998; Tokunaga *et al*, 1998), and therefore may be modulated by alterations in HIV-1 envelope function. Nef is important for the maintenance of high viral loads and progression to AIDS (Bour and Strebel, 2000; Fackler and Baur, 2002; Geyer *et al*, 2001), apart from rare exceptions (Churchill *et al*, 2004; Churchill *et al*, 2006a; Gorry *et al*, 2007a; Gorry *et al*, 2007b), but the contribution of specific Nef functions to pathogenesis in the immune system and CNS is unclear.

Nef down-modulates CD4 from the cell surface by associating CD4 with AP-1/AP-2 of clathrin-coated pits (Bresnahan *et al*, 1998; Greenberg *et al*, 1998) followed by transfer to lysosomes for degradation (Piguet *et al*, 1999). CD4 down-modulation enhances HIV-1 replication and infectivity, virion production and release, and prevents superinfection (Lama, 2003). Nef down-regulates MHC-I by targeting it to the endosome-to-Golgi sorting pathway (Doms and Trono, 2000), which protects infected cells from lysis mediated by HIV-1-specific CTLs (Collins *et al*, 1998). Because the CNS is an immune privileged site (Carson *et al*, 2006), the requirement for these immune evasion functions of Nef may be reduced during viral persistence in the brain.

The role of Nef in HIV-1 neuropathogenesis is unclear, and there have been only a few published studies of brain-derived *nef* alleles. Brain-derived *nef* alleles differentially induce pro-inflammatory gene expression in astrocytes (van Marle *et al*, 2004), suggesting a role for Nef in HAD pathogenesis. Moreover, a study that characterized *nef* alleles amplified directly from autopsy brain and matched lymph node from 2 subjects with late stage disease showed that CD4- and MHC-1 down-modulation activities are conserved among *nef* alleles derived from both tissue compartments, although the brain-derived *nef* alleles had a weaker ability to down-modulate MHC-1 (Agopian *et al*, 2007). Since there is reduced CTL surveillance within the brain, reduced MHC-1 down-modulation activity by brain-derived *nef* alleles may be an adaptive response due to reduced selection pressure to maintain this function of Nef. In this study, a brain-specific signature pattern of KEEE- or EKEE- at the PACS-1 binding site within *nef* contributed to the reduced MHC-1 down-modulation activities by these brain-derived *nef* alleles. Further studies by these investigators characterized *nef* alleles from primary virus isolates derived from the same tissues to

characterize the topology of a hydrophobic binding surface on Nef shown to be critical for the association between Nef and Pak2 (Agopian *et al*, 2007). Recently, Olivieri et al characterized *nef* alleles cloned directly from autopsy brain and lymphoid tissues of 4 subjects with HAD, and showed evidence for adaptive selection of *nef* sequences within brain (Olivieri *et al*). However, the function of these *nef* alleles was not examined.

To better understand functional characteristics of *nef* alleles isolated from CNS and other tissues, we characterized 82 *nef* alleles from brain-, spinal cord-, CSF-, and lymphoid tissue-derived HIV-1 viruses isolated from 7 subjects with HAD. CD4 and MHC-I down-regulation activities were highly conserved in this large panel of *nef* alleles, suggesting these functions are likely to contribute to HIV-1 replication and escape from immune control in the CNS.

Results and Discussion

CNS- and lymphoid tissue-derived primary HIV-1 isolates

To examine the CD4- and MHC-1 down-modulation activity of CNS-derived Nef proteins, we undertook a genetic and functional analysis of *nef* alleles cloned from a well characterized panel of primary CNS- and lymphoid tissue-derived HIV-1 viruses isolated from subjects with HAD (Gorry *et al*, 2001; Thomas *et al*, 2007). The viruses were isolated from brain, CSF, and PBMC of subject CB1 (CB1-br, -CSF, -PBMC), CSF, spinal cord and PBMC of subject CB3 (CB3-CSF, -SC, -PBMC), brain and spleen of subject MACS1 (MACS1-br, -Spln), brain and lymph node of subjects MACS2 and MACS3 (MACS2-br, -LN and MACS3-br, -LN, respectively), and brain of subjects UK1 and UK7 (UK1-br and UK7-br, respectively). The coreceptor usage, tropism and replication capacity of these primary HIV-1 isolates, and the clinical details of the study subjects from whom the viruses were isolated, have been described in detail previously (Gorry *et al*, 2001; Thomas *et al*, 2007). The clinical and neuropathological characteristics are summarized in Table 1. A different panel of *nef* alleles from brain and lymphoid tissues from subjects MACS2 and MACS3 was described by Agopian et al (Agopian *et al*, 2006; Agopian *et al*, 2007).

Nef protein expression of cloned *nef* alleles

HIV-1 *nef* alleles derived from the primary isolates were cloned into the pTarget expression plasmid, and those able to express Nef protein were identified by Western blotting (Figure 1). As controls, we cloned *nef* alleles from the reference HIV-1 strains NL4-3, 89.6, JRCSF and YU2 into pTarget, and confirmed by Western blotting that these reference *nef* alleles expressed detectable levels of Nef protein. With the exception of MACS2-br, 5 to 6 *nef* alleles capable of expressing Nef protein detectable by Western blotting were identified from each virus isolate (Figure 1). None of the *nef* alleles cloned from MACS2-br could express Nef protein, despite screening approximately 200 clones predicted to contain open *nef* reading frames (Figure 1, and data not shown). This finding is consistent with the study by Agopian et al, which reported that all *nef* alleles cloned from the MACS2-br isolate were defective due to a stop codon after 61 amino acids (Agopian *et al*, 2007). Thus, we established a large panel of primary *nef* alleles derived from well-characterized CNS- and lymphoid tissue-derived HIV-1 isolates from 7 patients with HAD.

Tissue-specific compartmentalization of *nef* alleles

The Nef clones were sequenced and analyzed for tissue-specific compartmentalization by phylogenetic analysis using a maximum likelihood algorithm. The consensus tree of 100 replicate data sets is shown in Figure 2. A multiple sequence alignment of unique *nef* sequences is shown in Figure 3. Sequences from each subject formed distinct monophyletic clusters. Analysis of intra-subject sequence sets demonstrated tissue-specific monophyletic clustering, indicating tissue-specific compartmentalization of the primary *nef* alleles. In addition, we found evidence of reduced genetic diversity among brain isolate-derived *nef* alleles compared to lymphoid tissue isolate-derived *nef* alleles for subjects MACS1 and MACS2, but not MACS3. These findings are consistent with previous studies of compartmentalization and genetic distance of tissue-derived *nef* alleles from these 3 subjects (Olivieri *et al*, 2010). In addition, the *nef* alleles derived from the primary virus isolates described here cluster with the respective tissue-derived *nef* alleles (data not shown), validating that they are representative of virus present *in vivo*. Although compartmentalization was evident, the sequence alterations that segregated the brain isolate- and lymphoid tissue isolate-derived *nef* alleles were strain-specific, and no signature sequence defining Nef-related neurotropism *per se* was identified.

Identification of brain-derived, defective *nef* alleles

To better understand the lack of Nef protein expression by *nef* alleles derived from MACS2-br, multiple sequence alignments were examined for sequence alterations (Figure 3). Sequence analysis revealed a conserved frame shift mutation in all MACS2-br *nef* clones that terminated the *nef* reading frame upstream of the CD4 and PACS-1 binding sites. Given that previous studies demonstrated intact *nef* alleles amplified directly from brain tissue of subject MACS2 (Agopian *et al*, 2007; Olivieri *et al*, 2010), we cannot conclude that Nef was not required for CNS infection or the development of HAD in this subject, as it is possible that a particular MACS2-br variant was selected in culture. On the other hand, since the primary MACS2-br isolate replicates to high levels in primary cells, including monocyte-derived macrophages and primary human microglia (Gorry *et al*, 2001), these results suggest that the presence of Nef is not required for replication of this isolate in these target cells *in vitro*. Interestingly, although the MACS2-br virus is highly macrophage tropic *in vitro*, this isolate does not induce significant levels of neuronal apoptosis in mixed human fetal brain cultures compared to other highly macrophage tropic primary HIV-1 isolates (Gorry *et al*, 2002), raising the possibility that Nef may enhance mechanisms distinct from viral replication that are important for HIV-1 neurovirulence.

CD4 down-modulatory activities of CNS- and lymphoid tissue-derived *nef* alleles

To better understand functions of these primary *nef* alleles, those with unique sequences (Figure 3) were further characterized for their ability to down-modulate cell surface CD4 expression. Previous studies on brain-derived *nef* alleles conducted similar studies by cotransfection of Nef- and GFP-expressing plasmids, and FACS analysis of GFP-positive cells (Agopian *et al*, 2007). To allow coexpression of Nef and a fluorescent marker in the same cells, we developed an assay in which *nef* alleles are subcloned into the pIRES2-ZsGreen1 expression plasmid upstream of an internal ribosome entry site which drives the

simultaneous expression of the ZsGreen1 fluorescent protein. Thus, expression of ZsGreen1 can be used as a specific marker for Nef-expressing cells.

The ability of the CNS- and lymphoid tissue-derived *nef* alleles to down-modulate cell surface CD4 expression was tested in Jurkat cells (Figure 4). Nef plasmid containing NL4-3 *nef* with the LL₁₆₄AA (LLAA) mutation that abolishes CD4 downmodulation but not MHC-1 downmodulation (Bresnahan *et al*, 1998; Greenberg *et al*, 1998), was subcloned into pIRES2-ZsGreen1 and included as a negative control. Transfection efficiency, based on ZsGreen1 expression, ranged from approximately 20 to 30% (data not shown). Residual CD4 expression in the ZsGreen1+ populations of Nef-transfected cells was expressed as a fraction of that measured in cells transfected with pIRES2-ZsGreen1 alone. The reference *nef* alleles NL4-3, 89.6, JRCSF and YU2 down-modulated cell surface CD4 expression on Jurkat cells to similar levels, whereas the LLAA mutant had no effect on cell surface CD4 expression (Figure 4A). Representative flow cytometry plots for cells transfected with vector alone, NL4-3 Nef, or the LLAA mutant, and then stained for CD4 expression are shown in Figure 4B. The CD4 down-modulation activity of the CNS- and lymphoid tissue virus-derived *nef* alleles is shown in Figure 4C. This function of Nef was largely conserved among the primary virus-derived *nef* alleles although particular clones, for example CB3-CSF-6 and MACS3-Br-5 were less efficient in down-modulating CD4 expression. Similar results were obtained using the JC53 cell line (data not shown). There was a significant decrease in the ability of MACS3-br *nef* alleles to down-modulate CD4 compared to MACS3-LN *nef* alleles. However, there was no consistent difference in CD4 down-modulatory activity between CNS- and lymphoid tissue virus-derived *nef* alleles. Thus, CD4 down-regulation was generally conserved among *nef* alleles from CNS-derived HIV-1 isolates.

MHC-1 down-modulatory activities of CNS- and lymphoid tissue-derived *nef* alleles

We next measured the ability of these *nef* alleles to down-modulate cell surface MHC-1 expression in Jurkat cells (Figure 5). Nef plasmid containing NL4-3 *nef* with the EEEE₆₅AAAA (E4A) mutation that abolishes MHC-1 down-modulation but not CD4 down-modulation (Piguet *et al*, 2000), was cloned into pIRES2-ZsGreen1 and included as a negative control. The reference *nef* alleles NL4-3, 89.6, JRCSF and YU2 down-modulated cell surface MHC-1 expression to similar levels, whereas the E4A mutant had no effect (Figure 5A). Representative flow cytometry plots for cells transfected with vector alone, NL4-3 Nef, or the E4A mutant and stained for MHC-1 expression are shown in Figure 5B. MHC-1 down-modulation activity of the CNS- and lymphoid tissue virus-derived *nef* alleles is shown in Figure 5C. Consistent with the results of the CD4 down-modulation studies, there was no consistent difference in MHC-1 down-modulatory activity between CNS- and lymphoid tissue virus-derived *nef* alleles with the exception of MACS3. Similar to the reduced ability of MACS3-br *nef* alleles to down-modulate CD4, these clones had a reduced ability to down-modulate MHC-1 compared to MACS3-LN *nef* alleles, consistent with a previous study that showed reduced MHC-1 down-modulation activity by *nef* alleles cloned directly from brain tissue of subject MACS3 (Agopian *et al*, 2007). Thus, MHC-I down-modulation activity was generally conserved among *nef* alleles from CNS-derived HIV-1 isolates.

Sequence alterations in *nef* associated with differences in CD4 and MHC-1 down-modulation

Nef sequences were analyzed to identify sequence alterations associated with reduced CD4 and/or MHC-1 down-modulatory activity (Figure 3). Both CB3-CSF clones analyzed for CD4 down-modulation (CB3-CSF-1 and -6) have conserved CD4 binding sites and motifs in the flexible loop that are critical for association with the endocytic machinery. However, CB3-CSF-6, which is defective in down-modulating CD4 (Figure 4), has a only a V30M mutation that distinguishes this clone from CB3-CSF-1, which may account for its diminished activity. MACS3-br *nef* alleles, which had reduced CD4 down-modulatory activity compared to MACS3-LN *nef* alleles (Figure 4), had conserved K₁₅₂Q mutations, a location that is in close proximity to the β -COP and adaptor protein complex (AP-1/2/3) binding motifs of Nef. Down-modulation of CD4 by Nef requires association with endocytic machinery components, which includes β -COP, AP-1/2/3 and V-ATPase, to target CD4 to lysosomes for degradation. Therefore genetic variants within this region may affect association with these components and thereby result in reduced CD4 down-modulation activity. MACS3-br *nef* alleles had distinguishing mutations within the PACS-1 motif; specifically, MACS3-br *nef* alleles had either EEEEE₆₆ or EEEG₆₅, whereas MACS3-LN *nef* alleles, which had stronger MHC-1 down-modulatory activity (Figure 5), had EQEE₆₅ with the exception of MACS3-LN-7 which had EQKE₆₅. The EQEE₆₅ PACS-1 binding motif has increased MHC-1 down-modulatory activity compared to the EEEE motif (Agopian *et al*, 2007), and therefore is likely to contribute to the stronger MHC-1 down-modulatory activity of MACS3-LN compared to MACS3-br *nef* alleles. Further mutagenesis studies are required to determine the functional significance of these genetic variants.

Conclusions

The results of our study demonstrate compartmentalisation of *nef* sequences derived from the CNS using autopsy tissue-derived HIV-1 viruses isolated from 7 subjects who died from AIDS with HAD. Consistent with a previous study (Olivieri *et al*, 2010), we found evidence of reduced *nef* sequence diversity in brain- compared to lymphoid tissue-derived isolates from 3 subjects, The predominance of defective *nef* alleles in one brain-derived HIV-1 isolate that replicates efficiently in PBMC, macrophages and microglia *in vitro* implies that Nef is not required for efficient HIV-1 replication in these cells *in vitro*. Nonetheless, the finding that the vast majority of *nef* alleles are intact when cloned directly from the same brain tissue sample rather than cloned from a viral isolate (Agopian *et al*, 2007), and when cloned directly from autopsy brain tissues from other late stage AIDS subjects (Olivieri *et al*, 2010) implies that Nef function is likely to be required for CNS infection *in vivo*. Moreover, data from the SIV model also suggest that Nef function is likely to be required for CNS infection (Thompson *et al*, 2003). Using a large panel of *nef* clones from CNS HIV-1 isolates derived from brain, CSF, and spinal cord, we demonstrated that CD4 and MHC-1 down-modulation activities are highly conserved functions of *nef* alleles derived from brain and other tissues. The CD4 down-modulation activity of Nef may be more relevant for the CD4-dependent infection of brain macrophage-lineage cells, rather than the CD4-independent infection of astrocytes. Further studies are required to determine the CD4 and MHC-1 down-modulation activities of primary *nef* alleles in primary fetal microglia, which

would further clarify the importance of Nef in HIV-1 neuropathogenesis. Our findings, along with those of Agopian et al (2007), provide evidence that these activities may be reduced in only a minority of HIV-1-infected patients, possibly reflecting viral adaptation in response to reduced CD4 levels and reduced immune surveillance in the CNS. Thus, CD4 and MHC-1 down-modulation are likely to be important functions of Nef within the CNS that contribute to viral replication in the CNS and the pathogenesis of HAD.

Materials and Methods

Primary HIV-1 isolates

The primary CNS- and lymphoid tissue-derived HIV-1 viruses isolated from subjects CB1, CB3, MACS1, MACS2, MACS3, UK1 and UK7 have been described in detail previously (Gorry *et al*, 2001; Thomas *et al*, 2007).

Plasmids

Plasmids containing the HIV-1 proviruses NL4-3, 89.6, JRCSF and YU2 have been described previously (Adachi *et al*, 1986; Collman *et al*, 1992; Koyanagi *et al*, 1987; Li *et al*, 1991). The Nef mutants E4A and LLAA have been described previously (Agopian *et al*, 2007).

Cell lines

293T cells were cultured in DMEM supplemented with 10% (vol/vol) fetal calf serum (FCS), and 100 µg of penicillin and streptomycin per ml. JC53 cells are derived from the HeLa cell line and stably express high levels of CD4, CXCR4 and CCR5 on the cell surface (Platt *et al*, 1998), and were cultured in DMEM supplemented with 10% (vol/vol) FCS, and 100 µg of penicillin and streptomycin per ml. The Jurkat T-cell line was cultured in RPMI medium supplemented with 10% (vol/vol) FCS, and 100 µg of penicillin and streptomycin per ml.

Nef cloning, sequencing and phylogenetic analysis

The *nef* coding region of the HIV-1 genome was amplified from cDNA generated from viral supernatants by reverse transcription PCR with SuperScript III reverse transcriptase (Invitrogen) and random hexamers, using high-fidelity DNA polymerase and primers NefLTR5' #2 (5'-TAACTTGCTCAATGCCACAGA-3') and NefLTR3' #2 (5'-AAAAGGGTCTGAGGGATCTCT-3'), which amplifies an approximately 1 kb fragment corresponding to nucleotides 8650 to 9685 of HIV-1 NL4-3. The cycling conditions consisted of an initial denaturation step of 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 72°C, and then a final extension step of 7 min at 72°C. The products of 3 independent PCR reactions were pooled and inserted into the pTarget expression plasmid (Promega, WI) by TA cloning. For the generation of reference Nef clones, the proviral plasmids of HIV-1 NL4-3, 89.6, JRCSF and YU2 were used as template DNA for PCR, and amplified products were similarly inserted into pTarget. The *nef* coding region was sequenced with an Epicenter SequiTherm EXCEL II sequencing kit (LiCor, NE, USA), and analyzed using a LiCor 4000 DNA Sequencer. Phylogenetic analysis was conducted using a maximum likelihood algorithm, as described previously (Gray *et al*,

2007). For CD4 and MHC-1 downmodulation assays, Nef clones with unique nucleotide sequences were subcloned into the pIRES2-ZsGreen1 expression plasmid (Clontech, CA, USA), upstream of an internal ribosome entry site which drives the simultaneous expression of Nef together with the ZsGreen1 fluorescent protein.

Nef Western blotting

293T cells were transfected with 4.0 µg of pTarget Nef plasmid using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Cell lysates were prepared 48 h later as described previously (Agopian *et al.*, 2007), and equal amounts of total protein were electrophoresed in 12% (wt/vol) SDS-PAGE gels and analyzed by Western blotting using sheep anti-Nef antisera as described previously (Gorry *et al.*, 1999).

CD4 and MHC-1 down-modulation assays

These assays were conducted as described in detail previously (Agopian *et al.*, 2007), except that Nef and the fluorescent ZsGreen1 protein were coexpressed by single transfection of pIRES2-ZsGreen1-Nef plasmids in Jurkat and JC53 cells. Transfection of Jurkat cells was achieved by electroporation of 7.5×10^6 cells with 7.5 µg DNA with a Biorad electroporator set at 250V, 950µF, and 200Ω. JC53 cells were transfected with 4 µg DNA using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol.

Nucleotide sequence accession numbers

The *nef* nucleotide sequences reported here have been assigned GenBank accession numbers HQ174334 to HQ174415.

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References

- Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol.* 1986; 59:284–291. [PubMed: 3016298]
- Agopian K, Wei BL, Garcia JV, Gabuzda D. A hydrophobic binding surface on the human immunodeficiency virus type 1 Nef core is critical for association with p21-activated kinase 2. *J Virol.* 2006; 80:3050–3061. [PubMed: 16501114]
- Agopian K, Wei BL, Garcia JV, Gabuzda D. CD4 and MHC-I downregulation are conserved in primary HIV-1 Nef alleles from brain and lymphoid tissues, but Pak2 activation is highly variable. *Virology.* 2007; 358:119–135. [PubMed: 16979207]
- Aiken C. Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporine A. *J Virol.* 1997; 71:5871–5877. [PubMed: 9223476]
- Bour S, Strebel K. HIV accessory proteins: multifunctional components of a complex system. *Adv Pharmacol.* 2000; 48:75–120. [PubMed: 10987089]

- Bresnahan PA, Yonemoto W, Ferrell S, Williams-Herman D, Geleziunas R, Greene WC. A dileucine motif in HIV-1 Nef acts as an internalization signal for CD4 downregulation and binds the AP-1 clathrin adaptor. *Curr Biol*. 1998; 8:1235–1238. [PubMed: 9811606]
- Carson MJ, Doose JM, Melchior B, Schmid CD, Ploix CC. CNS immune privilege: hiding in plain sight. *Immunol Rev*. 2006; 213:48–65. [PubMed: 16972896]
- Churchill M, Sterjovski J, Gray L, Cowley D, Chatfield C, Learmont J, Sullivan JS, Crowe SM, Mills J, Brew BJ, Wesselingh SL, McPhee DA, Gorry PR. Longitudinal analysis of nef/long terminal repeat-deleted HIV-1 in blood and cerebrospinal fluid of a long-term survivor who developed HIV-associated dementia. *J Infect Dis*. 2004; 190:2181–2186. [PubMed: 15551218]
- Churchill MJ, Figueiredo A, Cowley D, Gray L, Purcell DF, Sullivan JS, McPhee DA, Wesselingh SL, Brew BJ, Gorry PR. Transcriptional activity of blood-and cerebrospinal fluid-derived nef/long-terminal repeat sequences isolated from a slow progressor infected with nef-deleted human immunodeficiency virus type 1 (HIV-1) who developed HIV-associated dementia. *J Neurovirol*. 2006a; 12:219–228. [PubMed: 16877303]
- Churchill MJ, Gorry PR, Cowley D, Lal L, Sonza S, Purcell DF, Thompson KA, Gabuzda D, McArthur JC, Pardo CA, Wesselingh SL. Use of laser capture microdissection to detect integrated HIV-1 DNA in macrophages and astrocytes from autopsy brain tissues. *J Neurovirol*. 2006b; 12:146–152. [PubMed: 16798676]
- Churchill MJ, Wesselingh SL, Cowley D, Pardo CA, McArthur JC, Brew BJ, Gorry PR. Extensive astrocyte infection is prominent in human immunodeficiency virus-associated dementia. *Ann Neurol*. 2009; 66:253–258. [PubMed: 19743454]
- Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature*. 1998; 391:397–401. [PubMed: 9450757]
- Collman R, Balliet JW, Gregory SA, Friedman H, Kolson DL, Nathanson N, Srinivasan A. An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immunodeficiency virus type 1. *J Virol*. 1992; 66:7517–7521. [PubMed: 1433527]
- Craig HM, Pandori MW, Guatelli JC. Interaction of HIV-1 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity. *Proc Natl Acad Sci U S A*. 1998; 95:11229–11234. [PubMed: 9736718]
- Doms RW, Trono D. The plasma membrane as a combat zone in the HIV battlefield. *Genes Dev*. 2000; 14:2677–2688. [PubMed: 11069884]
- Fackler OT, Baur AS. Live and let die: Nef functions beyond HIV replication. *Immunity*. 2002; 16:493–497. [PubMed: 11970873]
- Geleziunas R, Xu W, Takeda K, Ichijo H, Greene WC. HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. *Nature*. 2001; 410:834–838. [PubMed: 11298454]
- Geyer M, Fackler OT, Peterlin BM. Structure--function relationships in HIV-1 Nef. *EMBO Rep*. 2001; 2:580–585. [PubMed: 11463741]
- Gonzalez-Scarano F, Martin-Garcia J. The neuropathogenesis of AIDS. *Nat Rev Immunol*. 2005; 5:69–81. [PubMed: 15630430]
- Gorry PR, Bristol G, Zack JA, Ritola K, Swanstrom R, Birch CJ, Bell JE, Bannert N, Crawford K, Wang H, Schols D, De Clercq E, Kunstman K, Wolinsky SM, Gabuzda D. Macrophage Tropism of Human Immunodeficiency Virus Type 1 Isolates from Brain and Lymphoid Tissues Predicts Neurotropism Independent of Coreceptor Specificity. *J Virol*. 2001; 75:10073–10089. [PubMed: 11581376]
- Gorry PR, Churchill M, Learmont J, Cherry C, Dyer WB, Wesselingh SL, Sullivan JS. Replication-dependent pathogenicity of attenuated nef-deleted HIV-1 in vivo. *J Acquir Immune Defic Syndr*. 2007a; 46:390–394. [PubMed: 17993857]
- Gorry PR, Howard JL, Churchill MJ, Anderson JL, Cunningham A, Adrian D, McPhee DA, Purcell DF. Diminished production of human immunodeficiency virus type 1 in astrocytes results from inefficient translation of gag, env, and nef mRNAs despite efficient expression of Tat and Rev. *J Virol*. 1999; 73:352–361. [PubMed: 9847339]

- Gorry PR, McPhee DA, Verity E, Dyer WB, Wesselingh SL, Learmont J, Sullivan JS, Roche M, Zaunders JJ, Gabuzda D, Crowe SM, Mills J, Lewin SR, Brew BJ, Cunningham AL, Churchill MJ. Pathogenicity and immunogenicity of attenuated, nef-deleted HIV-1 strains in vivo. *Retrovirology*. 2007b; 4:66. [PubMed: 17888184]
- Gorry PR, Taylor J, Holm GH, Mehle A, Morgan T, Cayabyab M, Farzan M, Wang H, Bell JE, Kunstman K, Moore JP, Wolinsky SM, Gabuzda D. Increased CCR5 affinity and reduced CCR5/CD4 dependence of a neurovirulent primary human immunodeficiency virus type 1 isolate. *J Virol*. 2002; 76:6277–6292. [PubMed: 12021361]
- Gray L, Churchill MJ, Sterjovski J, Witlox K, Learmont JC, Sullivan JS, Wesselingh SL, Gabuzda D, Cunningham AL, McPhee DA, Gorry PR. Phenotype and envelope gene diversity of nef-deleted HIV-1 isolated from long-term survivors infected from a single source. *Viol J*. 2007; 4:75. [PubMed: 17634131]
- Greenberg M, DeTulleo L, Rapoport I, Skowronski J, Kirchhausen T. A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for downregulation of CD4. *Curr Biol*. 1998; 8:1239–1242. [PubMed: 9811611]
- Koyanagi Y, Miles S, Mitsuyasu RT, Merrill JE, Vinters HV, Chen IS. Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. *Science*. 1987; 236:819–822. [PubMed: 3646751]
- Lama J. The physiological relevance of CD4 receptor down-modulation during HIV infection. *Curr HIV Res*. 2003; 1:167–184. [PubMed: 15043201]
- Lama J, Mangasarian A, Trono D. Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. *Curr Biol*. 1999; 9:622–631. [PubMed: 10375528]
- Li Y, Kappes JC, Conway JA, Price RW, Shaw GM, Hahn BH. Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication-competent and -defective viral genomes. *J Virol*. 1991; 65:3973–3985. [PubMed: 1830110]
- Luo T, Douglas JL, Livingston RL, Garcia JV. Infectivity enhancement by HIV-1 Nef is dependent on the pathway of virus entry: implications for HIV-based gene transfer systems. *Virology*. 1998; 241:224–233. [PubMed: 9499797]
- Olivieri KC, Agopian KA, Mukerji J, Gabuzda D. Evidence for adaptive evolution at the divergence between lymphoid and brain HIV-1 nef genes. *AIDS Res Hum Retroviruses*. 2010; 26:495–500. [PubMed: 20377428]
- Piguet V, Gu F, Foti M, Demareux N, Gruenberg J, Carpentier JL, Trono D. Nef-induced CD4 degradation: a diacidic-based motif in Nef functions as a lysosomal targeting signal through the binding of beta-COP in endosomes. *Cell*. 1999; 97:63–73. [PubMed: 10199403]
- Piguet V, Wan L, Borel C, Mangasarian A, Demareux N, Thomas G, Trono D. HIV-1 Nef protein binds to the cellular protein PACS-1 to downregulate class I major histocompatibility complexes. *Nat Cell Biol*. 2000; 2:163–167. [PubMed: 10707087]
- Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, Kabat D. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J Virol*. 1998; 72:2855–2864. [PubMed: 9525605]
- Thomas ER, Dunfee RL, Stanton J, Bogdan D, Taylor J, Kunstman K, Bell JE, Wolinsky SM, Gabuzda D. Macrophage entry mediated by HIV Envs from brain and lymphoid tissues is determined by the capacity to use low CD4 levels and overall efficiency of fusion. *Virology*. 2007; 360:105–119. [PubMed: 17084877]
- Thompson KA, Kent SJ, Gahan ME, Purcell DF, McLean CA, Preiss S, Dale CJ, Wesselingh SL. Decreased neurotropism of nef long terminal repeat (nef/LTR)-deleted simian immunodeficiency virus. *J Neurovirol*. 2003; 9:442–451. [PubMed: 12907389]
- Tokunaga K, Kojima A, Kurata T, Ikuta K, Inubushi R, Shimano R, Kawamura M, Akari H, Koyama AH, Adachi A. Producer cell-dependent requirement of the Nef protein for efficient entry of HIV-1 into cells. *Biochem Biophys Res Commun*. 1998; 250:565–568. [PubMed: 9784383]

van Marle G, Henry S, Todoruk T, Sullivan A, Silva C, Rourke SB, Holden J, McArthur JC, Gill MJ, Power C. Human immunodeficiency virus type 1 Nef protein mediates neural cell death: a neurotoxic role for IP-10. *Virology*. 2004; 329:302–318. [PubMed: 15518810]

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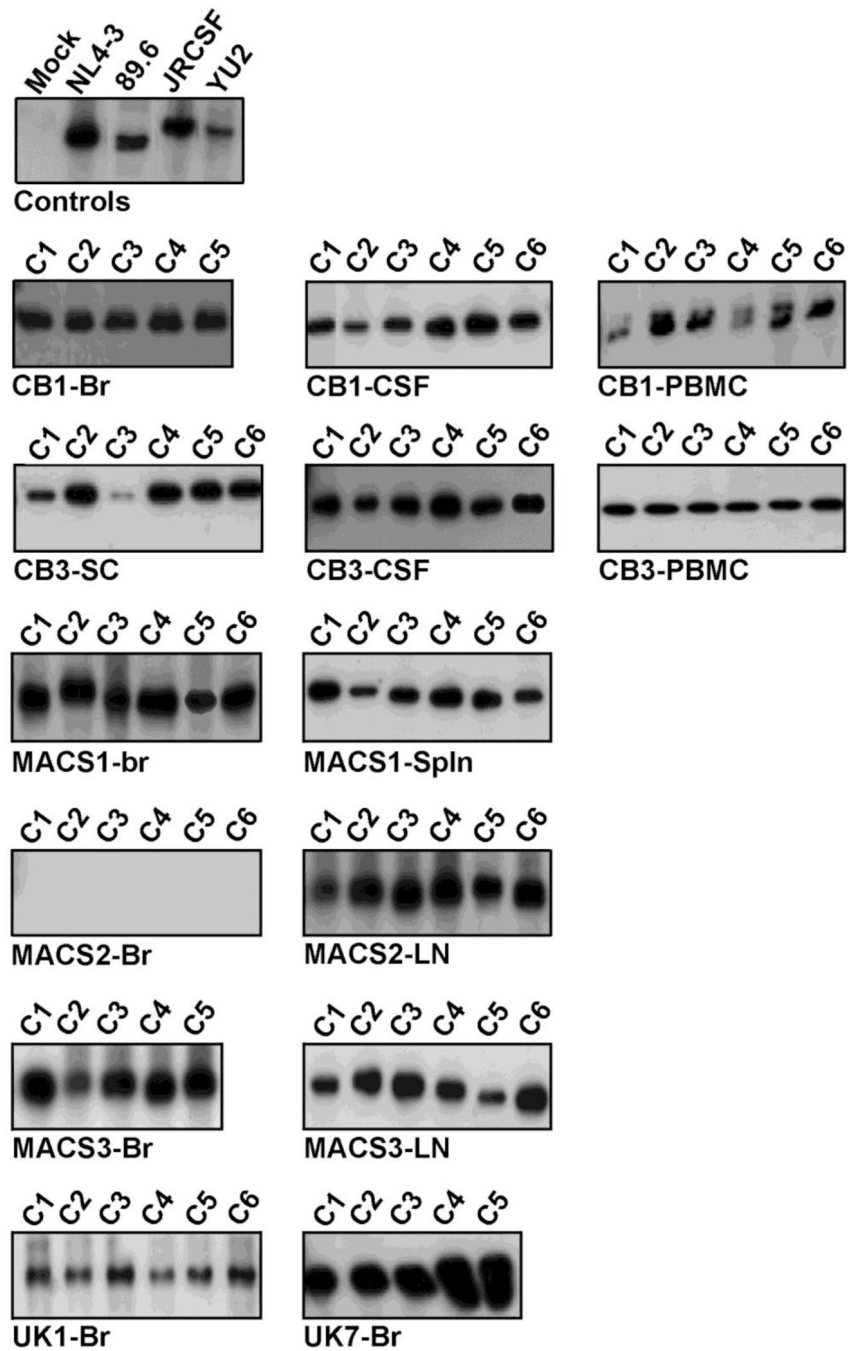


Figure 1. Western blot analysis of control and primary *nef* alleles
 Nef protein was detected by Western blot analysis of cell lysates derived from cells transfected with either control or primary *nef* alleles expressed from pTargetT, as described in Materials and Methods. C, clone number.

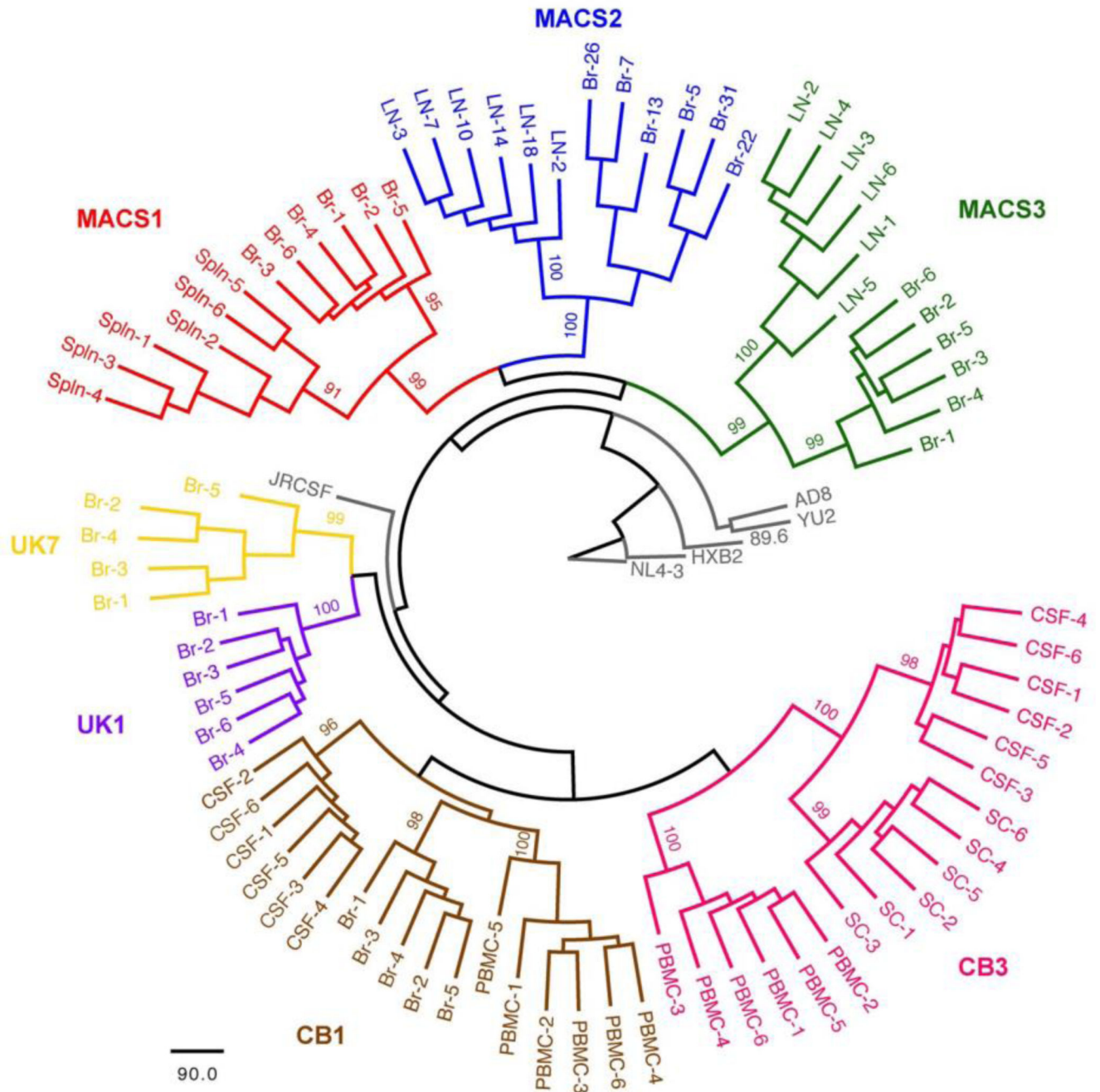


Figure 2. Phylogenetic analysis of *nef* nucleotide sequences derived from matched CNS- and lymphoid tissue HIV-1 isolates

nef sequences were amplified from CNS- and lymphoid tissue-derived virus isolates and are color coded by patient. The phylogenetic tree was constructed from a *nef* nucleotide multiple sequence alignment as described in Materials and Methods. Numbers associated with each branch are bootstrap values obtained from 100 replicates. Only values above 70 for the major branches are shown. Branch lengths are proportional to amount of sequence divergence. Clones are coded according to the tissue of origin and clone number. Br, brain;

LN, lymph node; CSF, cerebral spinal fluid; SC, spinal cord; Spln, spleen; PBMC, peripheral blood mononuclear cells. Control *nef* sequences are shown in grey.

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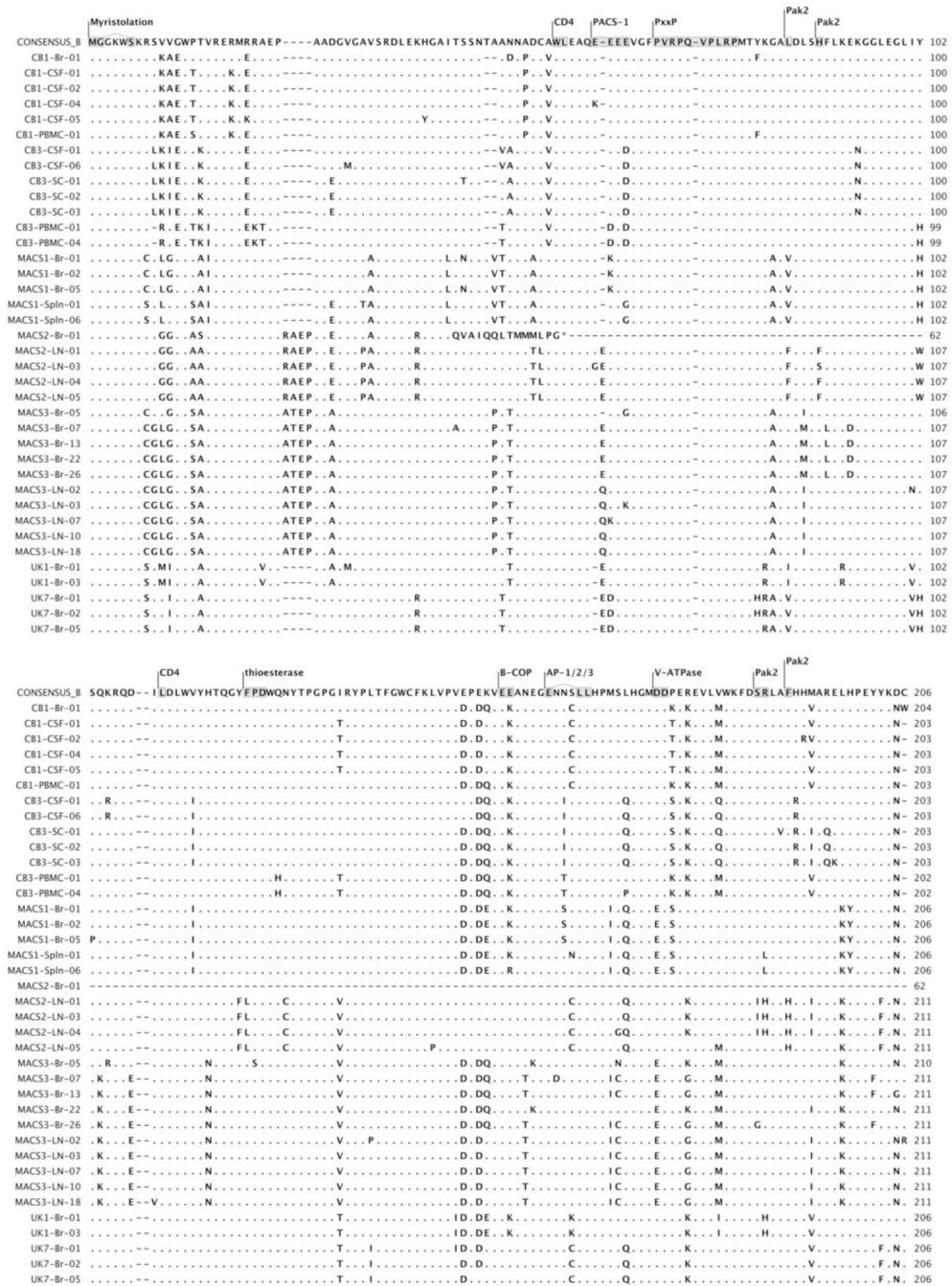


Figure 3. Nef amino acid sequences

Full-length HIV-1 Nef amino acid sequences were obtained from *nef* alleles cloned into pTargetT as described in Materials and Methods. Dots indicate residues identical to the clade B consensus sequence, and dashes indicate gaps. Star indicates frameshift mutation. The myristoylation signal, putative CD4 binding site, PACS-1, PxxP (SH3 binding domain), Pak2, thioesterase, β -COP, AP-1/2/3, and V-ATPase binding domains are annotated.

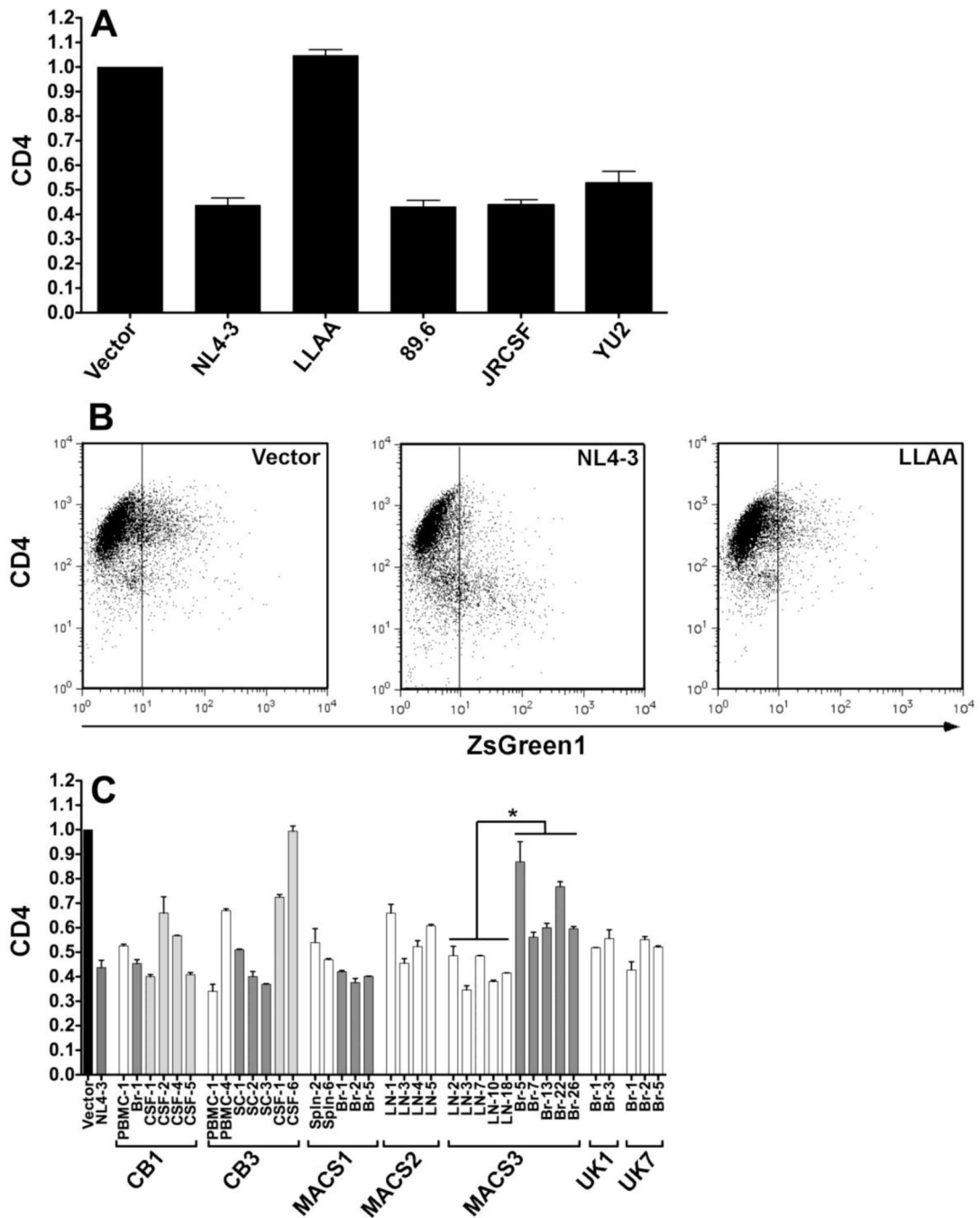


Figure 4. Down-modulation of cell surface CD4 by *nef* alleles derived from CNS- and lymphoid tissue HIV-1 isolates

Quantitation of flow cytometry analysis of cell surface CD4 on Jurkat cells transfected with either control *nef* plasmids (A) or primary *nef* alleles (C) as indicated. Relative surface CD4 expression was calculated from the geometric mean PE fluorescence in ZsGreen1-positive cells and is shown relative to ZsGreen1-positive vector-transfected cells. Examples of primary FACS data are shown in (B). The data shown are means of triplicates, and are representative of 3 independent experiments. Error bars represent standard deviations.

* $p < 0.01$; Statistical significance was calculated using a non-parametric Mann-Whiney U-test, using data medians from within the brain and lymph node groups of *nef* clones from subject MACS3.

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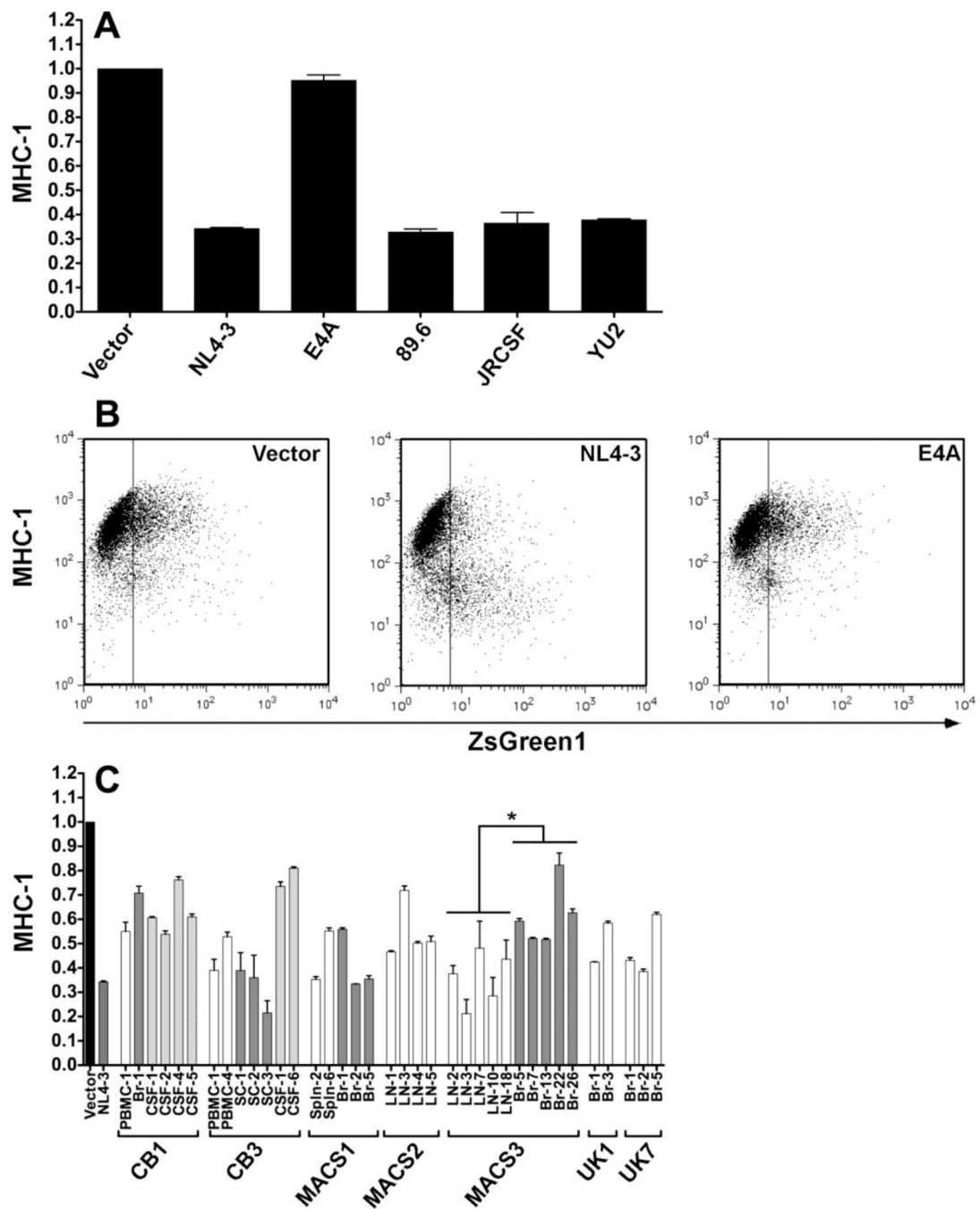


Figure 5. Down-modulation of cell surface MHC-1 by *nef* alleles derived from CNS- and lymphoid tissue HIV-1 isolates

Quantitation of flow cytometry analysis of cell surface MHC-1 on Jurkat cells transfected with either control *nef* plasmids (A) or primary *nef* alleles (C) as indicated. Relative surface MHC-1 expression was calculated from the geometric mean PE fluorescence in ZsGreen1-positive cells and is shown relative to ZsGreen1-positive vector-transfected cells. Examples of primary FACS data are shown in (B). The data shown are means of triplicates, and are representative of 3 independent experiments. Error bars represent standard deviations.

* $p < 0.01$; Statistical significance was calculated using a non-parametric Mann-Whiney U-test, using data medians from within the brain and lymph node groups of *nef* clones from subject MACS3.

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Table 1Clinical and neuropathological characteristics of the study subjects^a

Subject	Risk Factor ^b	Last CD4 count (cells/ μ l)	Antiretroviral(s)	HIV-1 encephalitis
CB1	MH	10	ddI (prior AZT)	Severe
CB3	MH	5	ddI (prior AZT and ddC)	Severe
MACS1	MH	2	None	Severe
MACS2	MH	52	AZT	Moderate
MACS3	MH	95	None	Moderate
UK1	IVDU	87	ddC (1 mo)	Moderate
UK7	IVDU	90	AZT	Severe

^aThese details have been published previously (Gorry *et al*, 2001; Thomas *et al*, 2007), and are summarized again here to assist in the interpretation of the Nef sequence and functional data.

^bMH, male homosexual; IVDU, intravenous drug user.