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

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Abstract Human immunodeficiency virus type 1 (HIV-1) *nef* undergoes adaptive evolution in the central nervous system (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 pathogenesis of HIV-associated dementia (HAD) is unclear. In this study, we characterized 82 *nef* alleles cloned from brain, cerebral spinal fluid, spinal cord, and blood/lymphoid tissue-derived HIV-1 isolates from seven 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 seven subjects (MACS2-br). The ability of Nef to down-modulate CD4 and MHC class 1 (MHC-1) was generally conserved among *nef* alleles from both CNS and lymphoid tissues. However, the potency of CD4 and MHC-1 down-modulation was variable, which was associated with sequence alterations known to influence these Nef functions. These results suggest that CD4 and MHC-1 down-modulations 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-1

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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, 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 down-modulation of CD4 and MHC class 1 (MHC-1) molecules from the cell surface, enhancement of viral replication and infectivity, and modulation of cellular signaling 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, 2006a; Gorry et al. 2007a, b), 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 and virion production and release and prevents superinfection (Lama 2003). Nef down-regulates MHC-1 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 Cytotoxic T-Lymphocytes (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 two 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 that is shown to be critical for the association between Nef and Pak2 (Agopian et al. 2007). Recently, Olivieri et al. (2010) characterized *nef* alleles cloned directly from autopsy brain and lymphoid tissues of four subjects with HAD and showed evidence for adaptive selection of *nef* sequences within the brain. However, the function of these *nef* alleles was not examined.

To better understand the functional characteristics of *nef* alleles isolated from CNS and other tissues, we characterized 82 *nef* alleles from brain-, spinal cord-, cerebral spinal fluid (CSF)-, and lymphoid tissue-derived HIV-1 viruses isolated from seven subjects with HAD. CD4 and MHC-1 down-regulation activities were highly conserved in this large panel of *nef* alleles, suggesting that 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 peripheral blood mononuclear cells (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 co-receptor 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. (2006, 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 (Fig. 1). As controls, we cloned *nef* alleles from the reference HIV-1

Table 1 Clinical and neuropathological characteristics of the study subjects

Subject	Risk Factor	Last CD4 count (cells/μl)	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

These 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

MH male homosexual, *IVDU* intravenous drug user, *mo* month, *ddI* didanosine, *AZT* zidovudine, *ddC* zalcitabine

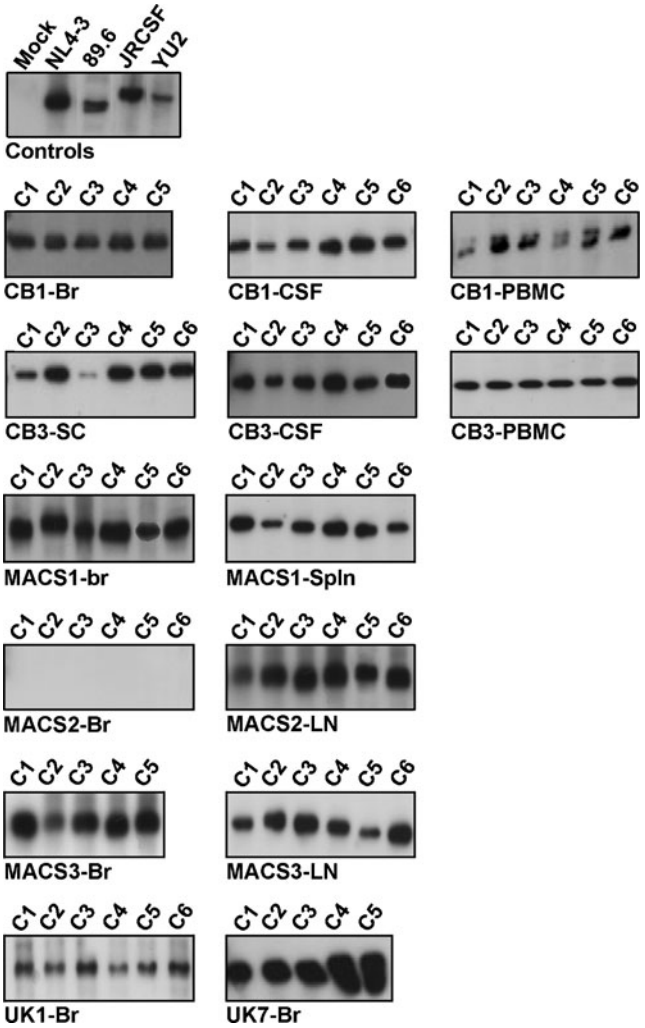

Fig. 1 Western blot analysis of control and primary nef alleles. Nef protein was detected by western blot analysis of cell lysates derived from cells transfected with either control or primary *nef* alleles expressed from pTargeT, as described in the Materials and Methods section. C, clone number

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, five to six *nef* alleles capable of expressing Nef protein detectable by western blotting were identified from each virus isolate (Fig. 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 (Fig. 1 and data not shown). This finding is consistent with the study by Agopian et al. (2007), which reported that all *nef* alleles cloned from the MACS2-br isolate were defective due to a stop codon after 61 amino acids. Thus, we established a large panel of primary *nef* alleles derived from well-characterized CNS- and lymphoid tissue-derived HIV-1 isolates from seven 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 Fig. 2. A multiple sequence alignment of unique *nef* sequences is shown in Fig. 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 three 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

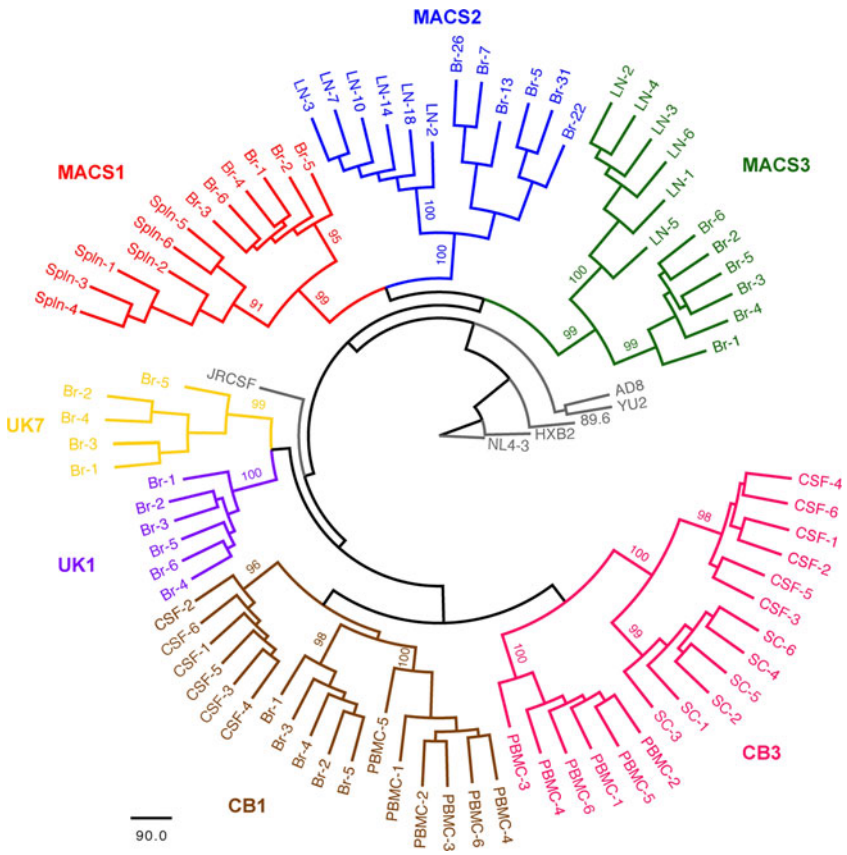

Fig. 2 Phylogenetic analysis of *nef* nucleotide sequences derived from matched CNS- and lymphoid tissue HIV-1 isolates. *nef* sequences were amplified from CNS- and lymphoid tissue-derived virus isolates and are color coded by patient. The phylogenetic tree was constructed from a *nef* nucleotide multiple sequence alignment as described in the Materials and Methods section. 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*

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 (Fig. 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 (Fig. 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 co-transfection of Nef- and Green Fluorescent Protein (GFP)-expressing plasmids and Fluorescence Activated Cell Sorting (FACS) analysis of GFP-positive cells (Agopian et al. 2007). To allow co-expression 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 (Fig. 4). Nef plasmid containing NL4-3 *nef* with the $LL_{164}AA$ (LLAA) mutation that abolishes

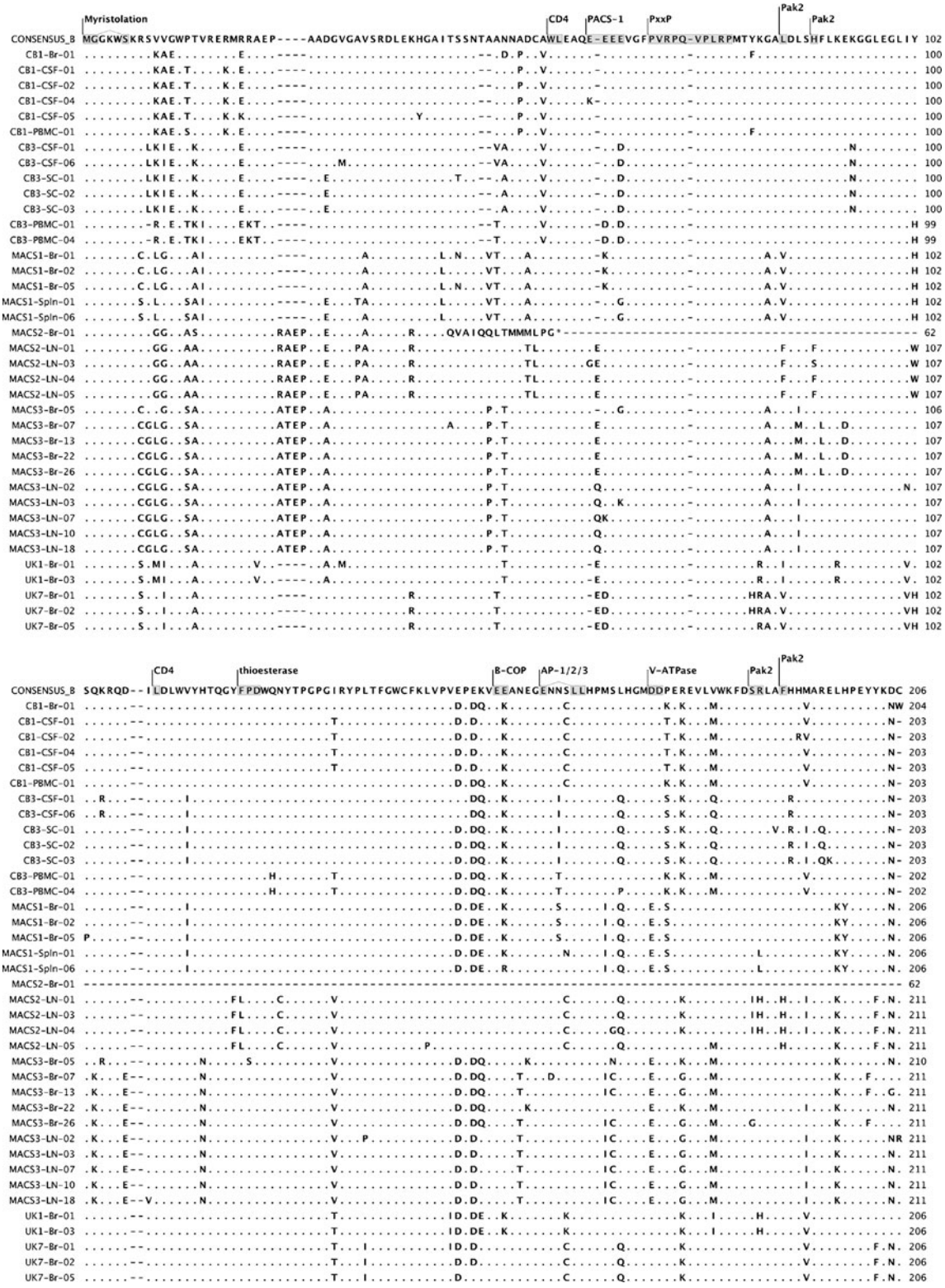

Fig. 3 Nef amino acid sequences. Full-length HIV-1 Nef amino acid sequences were obtained from *nef* alleles cloned into pTargeT as described in the Materials and Methods section. *Dots* indicate residues identical to the clade B consensus sequence, and *dashes* indicate gaps. *Star* indicates frameshift 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

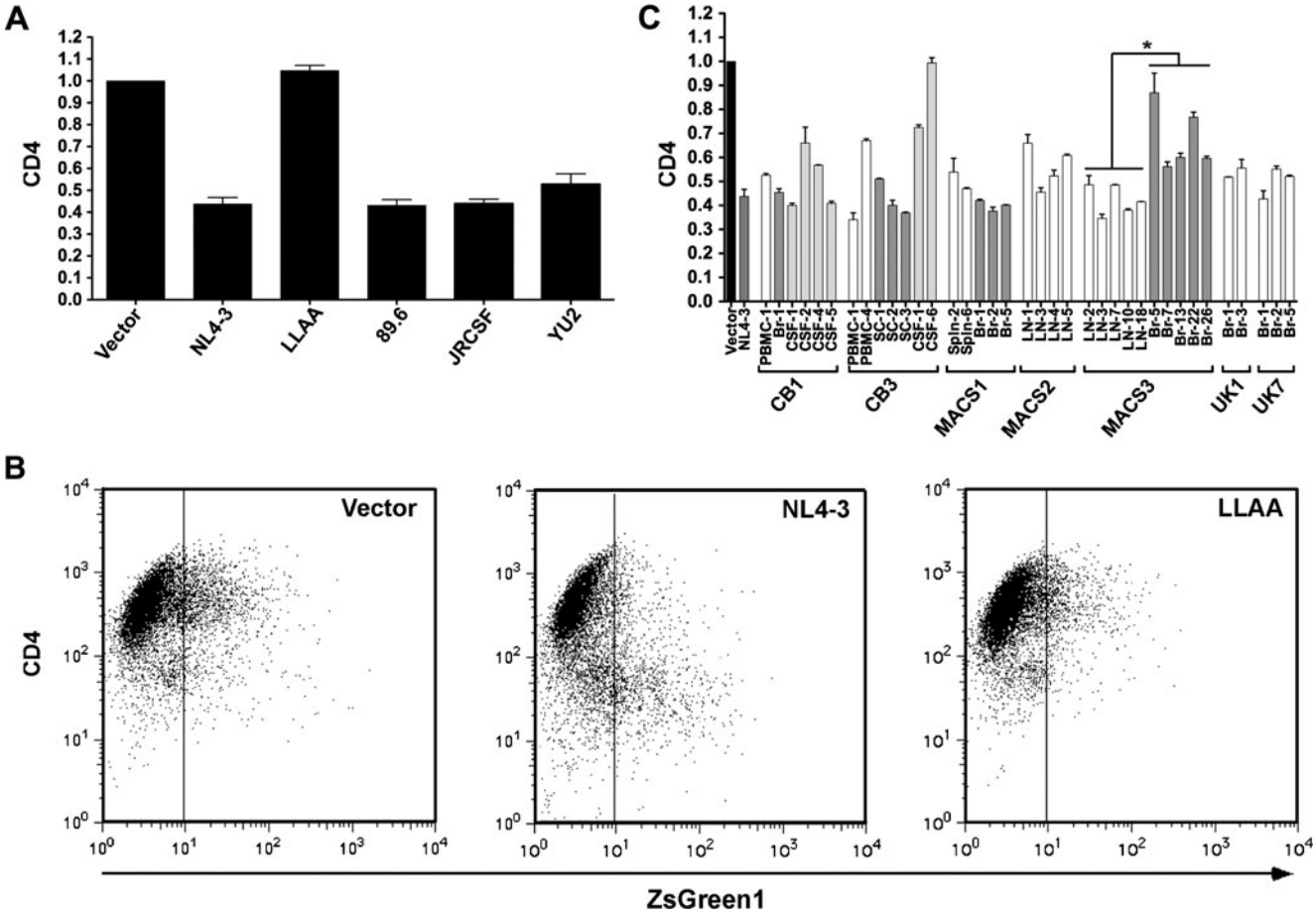

Fig. 4 Down-modulation of cell surface CD4 by *nef* alleles derived from CNS- and lymphoid tissue HIV-1 isolates. Quantitation of flow cytometry analysis of cell surface CD4 on Jurkat cells transfected with either control *nef* plasmids (**a**) or primary *nef* alleles (**c**) as indicated. Relative surface CD4 expression was calculated from the geometric mean phycoerythrin (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 three 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

CD4 down-modulation, but not MHC-1 down-modulation (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 (Fig. 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 Fig. 4b. The CD4 down-modulation activity of the CNS and lymphoid tissue virus-derived *nef* alleles is shown in Fig. 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 (Fig. 5). Nef plasmid containing NL4-3 *nef* with the $EEEE_{65}AAAA$ (E4A) mutation that

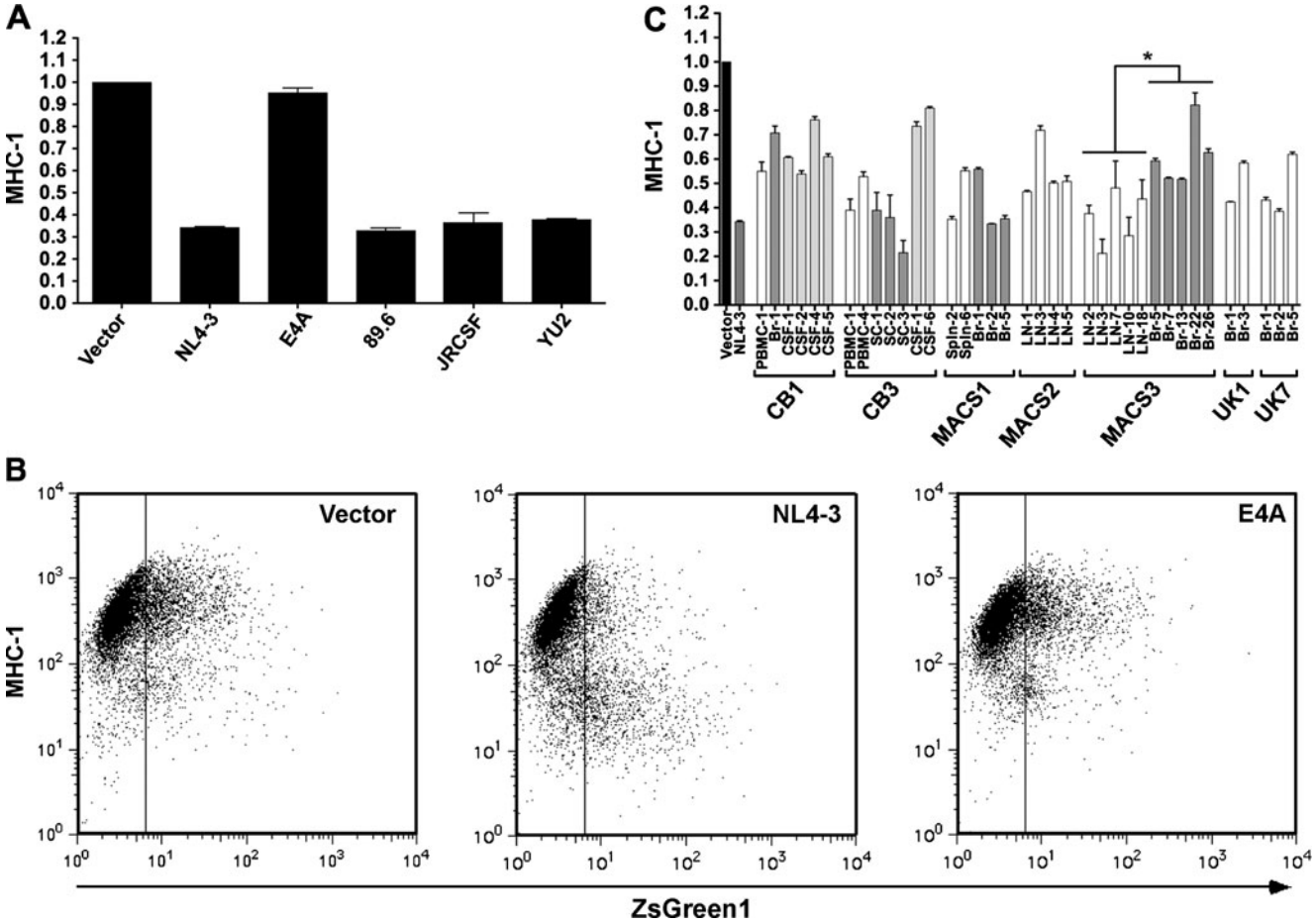

Fig. 5 Down-modulation of cell surface MHC-1 by *nef* alleles derived from CNS- and lymphoid tissue HIV-1 isolates. Quantitation of flow cytometry analysis of cell surface MHC-1 on Jurkat cells transfected with either control *nef* plasmids (**a**) or primary *nef* alleles (**c**) as indicated. Relative 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 three 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

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 (Fig. 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 Fig. 5b. MHC-1 down-modulation activity of the CNS and lymphoid tissue virus-derived *nef* alleles is shown in Fig. 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-1 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 (Fig. 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 (Fig. 4), has only a $V_{30}M$ 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 (Fig. 4), had conserved $K_{152}Q$ 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 $EEEEE_{66}$ or $EEEG_{65}$, whereas MACS3-LN *nef* alleles, which had stronger MHC-1 down-modulatory activity (Fig. 5), had $EQEE_{65}$ with the exception of MACS3-LN-7 which had $EQKE_{65}$. The $EQEE_{65}$ 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 the compartmentalization of *nef* sequences derived from the CNS using autopsy tissue-derived HIV-1 viruses isolated from seven 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-derived isolates compared with lymphoid tissue-derived isolates from three 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 Simian Immunodeficiency Virus (SIV) model also suggest that Nef function is likely 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 also been described previously (Agopian et al. 2007).

Cell lines 293T cells were cultured in DMEM supplemented with 10% (*v*/*v*) fetal calf serum (FCS) and 100 μg of penicillin and streptomycin per milliliter. 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 they were cultured in DMEM supplemented with 10% (*v*/*v*) FCS and 100 μg of penicillin and streptomycin per milliliter. The Jurkat T-cell line was cultured in RPMI medium supplemented with 10% (*v*/*v*) FCS and 100 μg of penicillin and streptomycin per milliliter.

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 three independent PCR reactions were pooled and inserted into the pTargeT expression plasmid (Promega, WI, USA) 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 down-modulation assays, Nef clones with unique nucleotide sequences were subcloned into the pIRES2-ZsGreen1 expression plasmid (Clonetech, 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% (*w*/*v*) 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 co-expressed 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 250 V, 950 μF, and 200 Ω. 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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