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Genetic and functional heterogeneity of CNS-derived *tat alleles* from patients with HIV-associated dementia

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Abstract Human immunodeficiency virus type 1 (HIV-1) demonstrates a high degree of viral diversity which has an impact on viral fitness. Genetic compartmentalization of HIV-1 proteins between central nervous system (CNS) and lymphoid tissues is well established and reflects altered requirements for HIV-1 replication in macrophages/microglia, brain-specific immune selection pressures and possibly the timing of virus invasion of the CNS. Tat-encoding mRNA has been detected in the CNS of HIV-1 infected individuals and its neurotoxic effects in the CNS are well documented. However, while CNS-derived *tat* sequences have demonstrated significant diversity, the effect of this molecular diversity on transcriptional regulation and its impact on the pathogenesis of HIV-associated dementia (HAD) remains unclear. In this study, we cloned and characterised 44 unique *tat* alleles from brain, cerebral spinal fluid, spinal cord and blood/lymphoid tissue-derived HIV-1 isolates from five subjects with HAD. While phylogenetic analyses revealed tissue-specific compartmentalization of Tat variants for two patients, broad compartmentalization across the panel of tissue-derived viruses was not observed. Despite the lack of consistent tissue-specific compartmentalization, sequence variations within patients segregated CNS and non-CNS *tat* alleles. These amino acid alterations predominated within the transactivation domain of Tat and could account for alterations in the ability of particular Tat proteins to transactivate the LTR. Although a subset of patients demonstrated reduced transactivation capacity among CNS-derived Tat proteins compared to those from matched lymphoid tissues, overall Tat proteins from the CNS to lymphoid compartments maintained similar levels of transactivation function. Together, these data suggest that despite the observed heterogeneity in *tat* alleles isolated from matched lymphoid to CNS compartments, Tat function is maintained, highlighting the importance of Tat function in HIV-1 neuropathogenesis.

Keywords HIV-1 · Dementia · Transactivation · CNS · Lymphoid

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Introduction

Human immunodeficiency virus type 1 (HIV-1) penetrates the central nervous system (CNS) and frequently causes neurological complications such as HIV-1 encephalitis (HIVE), HIV-1-associated dementia (HAD), and less severe forms of neurological impairment (Antinori et al. 2007). Perivascular macrophages and microglia support productive infection within the CNS (Cosenza et al. 2002; Gonzalez-Scarano and Martin-Garcia 2005; Williams et al. 2001). In contrast, whilst astrocytes contain integrated HIV-1 (Churchill et al.

2006) and are extensively infected *in vivo* (Churchill et al. 2009), they undergo a restricted non-productive infection (Tornatore et al. 1991). Multiple mechanisms involved in the restricted virus production in astrocytes have been described [reviewed in (Gorry et al. 2003)], including downregulation of long terminal repeat (LTR) activity (Niikura et al. 1996) and alterations in Tat-mediated transcription of viral genes (Taylor et al. 1992, 1993). The pathogenesis of HIV-1-associated neurological complications remains to be fully elucidated, although both host and viral mechanisms have been described (Gonzalez-Scarano and Martin-Garcia 2005).

HIV-1 demonstrates a high degree of viral diversity, which imparts an effect on viral replication fitness (Andreoni 2004). However, despite inter- and intra-patient genetic variation of HIV-1, we and other investigators have previously shown compartmentalization of HIV-1 viral proteins between CNS and lymphoid tissues (Gorry et al. 2001; Gray et al. 2009; Ohagen et al. 2003; Smit et al. 2001; Thompson et al. 2004) [reviewed in (Gonzalez-Scarano and Martin-Garcia 2005)]. The genetic compartmentalization of viral variants within the CNS suggest that adaptive changes may occur in response to the unique constraints within the CNS microenvironment, such as different target cell population and immune selection pressures, or may also reflect the timing of virus invasion of the CNS.

The HIV-1 regulatory protein Tat is encoded by two exons, expressed as either a 72 amino acid one-exon variant or 86–101 amino acid two-exon variant through alternative splicing (Frankel et al. 1989; Garcia et al. 1988). The key function of Tat is the regulation of HIV-1 transcription (Dayton et al. 1986), through its binding to the HIV-1 transactivation response element (TAR), an RNA hairpin structure which forms in the 5′ viral LTR (Rana and Jeang 1999). This interaction, and the subsequent binding and recruitment of several other cellular factors to the LTR, yields the phosphorylation of the C-terminal domain of RNA polymerase II greatly enhancing it processivity and therefore viral transcription (Herrmann and Rice 1993, 1995). Tat also plays a central role in HIV-1 neuropathogenesis through both neurotoxic (Eugenin et al. 2007; Mishra et al. 2008; Nath et al. 1996) and chemotactic (Albini et al. 1998) domains encoded within the first exon, and its ability to induce and modulate expression of host cytokines and neurotoxins (Buscemi et al. 2007; Nath et al. 1999).

Tat-encoding mRNA has been detected in the CNS of HAD and non-demented HIV-1/AIDS patients (Hudson et al. 2000; Wesselingh et al. 1993). Tat protein has also been detected in perivascular brain macrophages in AIDS patients (Del Valle et al. 2000; Hofman et al. 1999). Additionally, latently infected astrocytes have been shown to accumulate Tat mRNA transcripts (Bagasra et al. 1996; Tornatore et al. 1994) and secrete Tat protein (Brack-Werner, 1999). Brain derived *tat* sequences have demonstrated significant diversity, with variable effects on the modulation of host cytokines (Mayne et al. 1998) and LTR transactivation (Boven et al. 2007). However, the effect of this molecular diversity on the regulation of viral transcription within the CNS and its impact on the pathogenesis of HAD remains unclear. The present study sought to genetically and functionally characterise HIV-1 Tat derived from patients with HAD. To this end, we sought to establish and characterise a bank of unique HIV-1 Tat clones from matched CNS to lymphoid tissue derived HIV-1 isolates from patients with HAD.

Results

CNS- and lymphoid tissue-derived primary HIV-1 isolates To further understand the genetic and functional properties of Tat proteins isolated from the CNS, *tat* genes were cloned from a well-characterised panel of primary HIV-1 viruses isolated from autopsy CNS and lymphoid tissues of AIDS patients who died with dementia and HIVE. These viruses have been described in detail previously (Gorry et al. 2001, 2002), including the viral phenotypes and clinical characteristics of the subjects from whom they were isolated. The viruses used in this study were isolated from brain (BR), cerebral spinal fluid (CSF) and PBMC from subject CB1 (CB1-BR, -CSF, -PBMC) CSF, spinal cord (SC) and PBMC of subject CB3 (CB3-CSF, -SC, -PBMC), brain and spleen (SPLN) of subject MACS1 (MACS1-BR, -SPLN), and brain and lymph node (LN) of subjects MACS2 and MACS3 (MACS2-BR, -LN and MACS3-BR, -LN respectively). Brain tissue samples were collected from frontal lobe.

Establishment of a panel of unique CNS- and lymphoid-derived Tat variants To enable the genetic and functional characterisation of HIV-1 Tat from the CNS to lymphoid viral isolates, the first exon of HIV-1 *tat* was amplified and cloned into a mammalian expression vector. A 255-bp fragment spanning the first exon of HIV-1 *tat* (corresponding to nucleotides 5809–6064 of HIV-1 NL4.3) was amplified from viral supernatants, using reverse transcriptase-polymerase chain reaction (RT-PCR) with a high-fidelity DNA polymerase and nested PCR primers and cloned into pTargeT. A total of 283 recombinants were screened via PCR, resulting in 131 that were verified as recombinant Tat clones. Of these, 85 were sequenced and a total of 46 unique *tat* alleles were identified based on unique nucleotide sequences. Similarly, we cloned *tat* alleles from the reference strains YU2, JRCSF, AD8, HXB2 to NL43 as controls.

tat alleles failed to show clear tissue-specific compartmentalization The *tat* alleles from CNS to lymphoid tissue-derived viruses were sequenced and analysed for tissue-specific compartmentalization by neighbour-joining phylogenetic analysis (Fig. 1). The relative frequency of amino acid substitutions were compared to published clade B Tat sequences deposited in the Los Alamos HIV-1 Sequence Database (http://www.hiv.lanl.gov/). Phylogenetic analysis revealed *tat* sequences clustered according to patient. Distinct genetic compartmentalization was demonstrated for patients MACS3 and CB3, with significant bootstrap values separating sequences derived from CNS to lymphoid tissue viruses. In contrast, *tat* sequences from MACS1, MACS2 and CB1, whilst clustering within distinct patient groups, failed to demonstrate sequence segregation between compartments. Thus, patient-specific *tat* sequences were obtained, with a subset of patients demonstrating tissue specific compartmentalization.

Tat proteins from CNS- to lymphoid-derived viruses demonstrate a high degree of sequence heterogeneity Amino acid sequence alignments were performed to determine the presence of signature alterations that distinguish CNS and lymphoid tissue Tat proteins, or the presence of sequence changes within previously characterised functional domains which may impact Tat function. Multiple unique Tat clones were obtained for the majority of viral isolates, with the exception of CB3-CSF in which a single unique variant was dominant (Fig. 2). Analysis of Tat sequences revealed complete open-reading frames for 44 of the 45 unique clones, with a single clone from PBMC of patient CB1 having an in-frame stop codon. This clone is truncated at amino acid 11 and is predicted to be defective due to a lack of functional domains necessary for transactivation. Thus, a panel of 44 unique and potentially functional Tat clones was generated from the primary CNS- to lymphoid-derived viral isolates.

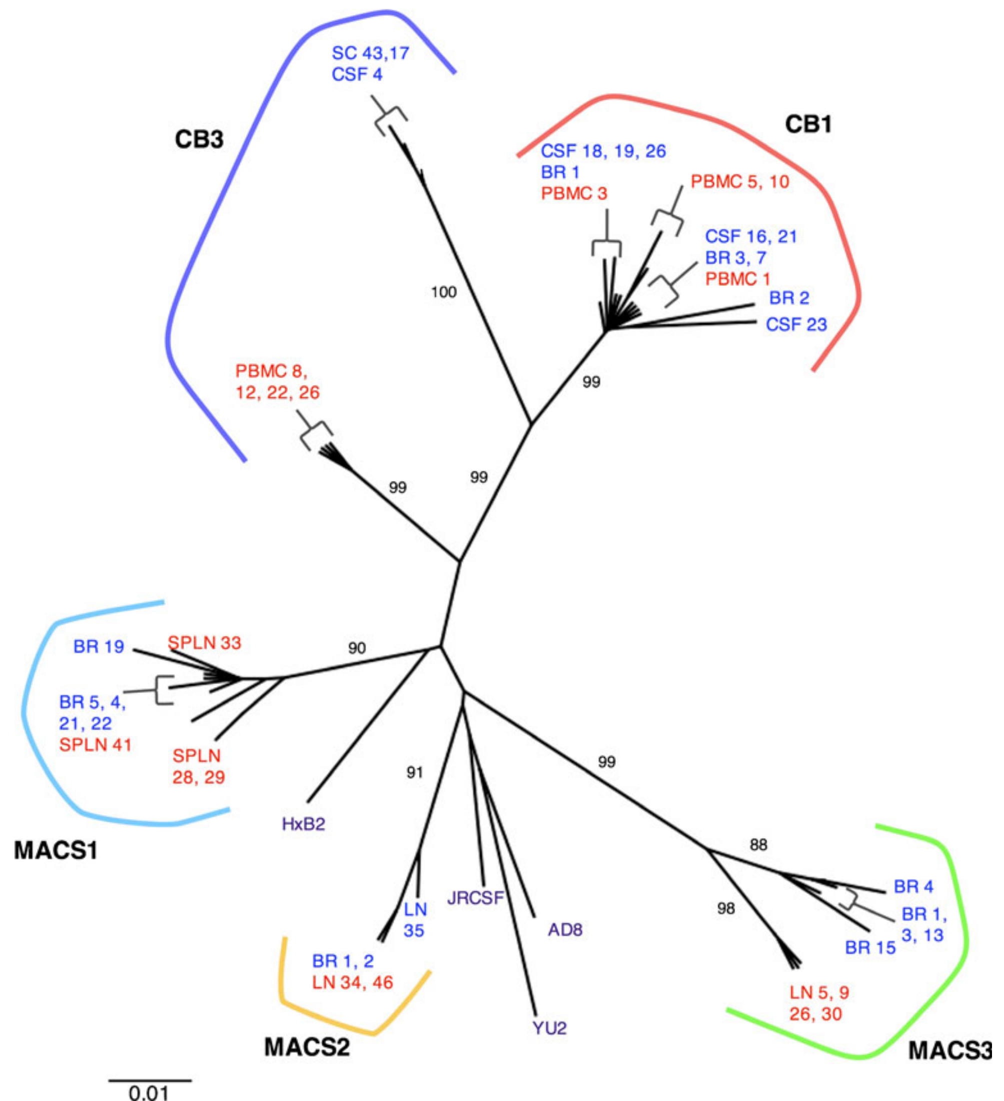

Fig. 1 Phylogenetic analysis of HIV-1 *tat* nucleotide sequences. Neighbour-joining phylogenetic analysis was performed on *tat* nucleotide sequences from matched CNS- to lymphoid-derived viral isolates. CNS-derived sequences are shown in *blue*, lymphoid-derived sequence are shown in *red* and control sequences in *purple*. Numbers associated with each branch are bootstrap values that represent the number of trees out of 100 replicates performed in which the same branching order was found. Only values for the major branches with significant bootstrap values are shown. Branch lengths are proportional to the amount of sequence divergence. *Scale bar* represents 1% sequence divergence

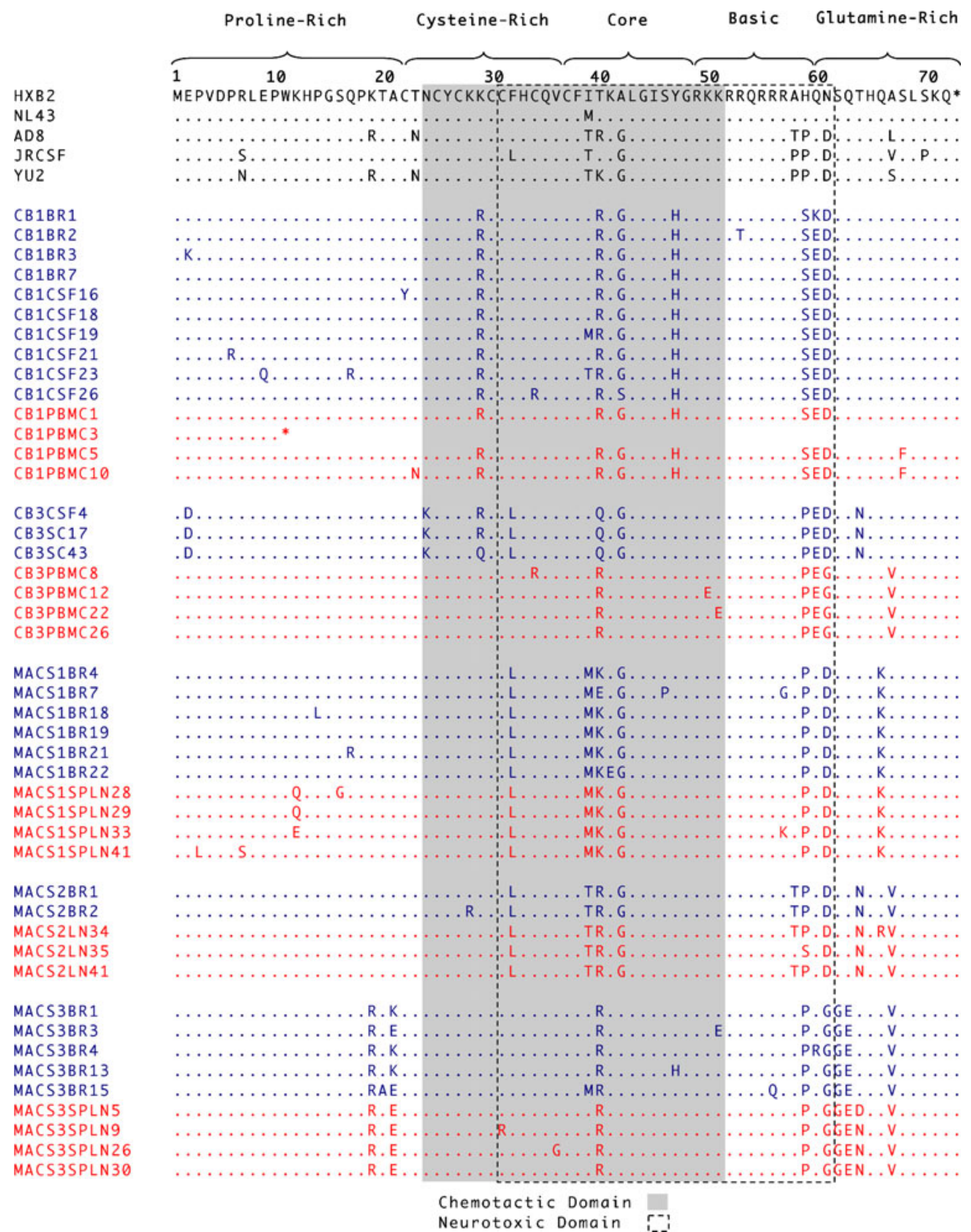

Fig. 2 Tat amino acid sequences. Full-length HIV-1 Tat amino acid sequences were obtained from *tat* alleles cloned into pTargeT as described in "Materials and methods" section. *Dots* indicate residues identical to the clade B consensus sequence. *Star* indicates premature stop codon. The proline-rich, cysteine-rich, core, basic and glutamine-rich domains are indicated. *Boxed areas* represent regions important for Tat's transactivation, chemoattractant and neurotoxic activities

Amino acid alterations associated with Tat function Multiple patient-specific amino acid substitutions were observed, which segregated CNS and lymphoid Tat sequences (Fig. 2). Regions of the Tat protein responsible for its chemoattractant (amino acids 24–51) and neurotoxic (amino acids 31–61) functions have previously been identified (Albini et al. 1998; Nath et al. 1996; Table 1). A number of mutations were observed in both the overlapping chemoattractant/neurotoxic and neurotoxic alone domains. For subject CB1, mutations in brain clones were observed at C22Y, C34R, and Y47H, with C22Y mapping to the neurotoxic domain, C34R and Y47H mapping to the overlapping chemoattractant/neurotoxic domains. PBMC-derived clones from the same subject also carried a mutation at Y47H. For subject CB3, all mutations in this functional region were seen in the PBMC-derived clones and mapped to the overlapping functional regions (K50E, K51E, C34R). Similarly, for the remaining subjects, all Tat mutations in this region mapped to the overlapping chemoattractant/neurotoxic domains (MACS1-BR :S46P, K41E, MACS 2-BR: K28R, MACS3-BR: K51E, Y47H, MACS 3-LN: C31R).

To assess the relative frequency and significance of the amino acid substitutions, an examination of clade B HIV-1 Tat sequences (n=756) deposited into the Los Alamos HIV-1 sequence database was performed (Table 1). The analysis was performed with an emphasis on sequence changes in the key functional cysteine rich, core and basic domains

Table 1 Frequency and significance of amino acid substitutions in CNS and lymphoid Tat

Virus	Clone number(s)	Clone frequency	Amino acid substitution	Description	% Frequency in clade B HIV-1 (n=756)	Reference
CB1-BR	1,2,3,7	4/4	Y47H	Y47H associated with reduced transactivation	1.7	(Kuppuswamy et al. 1989; Verhoef and Berkhout 1999)
CB1-BR	3	1/4	E2K	E2 provides intramolecular hydrogen bond with K51 and R53	0.7	(Pantano et al. 2004)
CB1-CSF	16,18,19,21,23,26	6/6	Y47H	Y47H associated with decreased transactivation activity	1.7	(Kuppuswamy et al. 1989; Verhoef and Berkhout 1999)
CB1-CSF	16	1/6	C22Y	C22 required for transactivation	0.7	(Kuppuswamy et al. 1989; Rice and Carlotti 1990a)
CB1-CSF	26	1/6	C34R	C34 required for transactivation	0	(Kuppuswamy et al. 1989; Rice and Carlotti 1990a)
CB1-PBMC	1,3,5,10	3/4	Y47H	Y47H associated with decreased transactivation activity	1.7	(Kuppuswamy et al. 1989; Verhoef and Berkhout 1999)
CB1-PBMC	3	1/4	W11*	Stop codon, truncated Tat protein	0	n/a
CB3-CSF	4	1/1	T64N	T64 site of phosphorylation by PKR, associated with decreased TAR binding	15	(Endo-Munoz et al. 2005)
CB3-SC	17,43	2/2	T64N	T64 site of phosphorylation by PKR, associated with decreased TAR binding	15	(Endo-Munoz et al. 2005)
CB3-PBMC	8	1/4	K50E	K50 site of Tat acetylation by p300/PCAF, potentially decreased transactivation	0	(Kiernan et al. 1999)
CB3-PBMC	12	1/4	K51E	K50 site of Tat acetylation by p300/PCAF, potentially decreased transactivation	0	(Kiernan et al. 1999)
CB3-PBMC	26	1/4	C34R	C34 required for transactivation	0	(Kuppuswamy et al. 1989; Rice and Carlotti 1990a)
MACS1-BR	18	1/6	S46P	S46 site of phosphorylation by CDK2, associated with reduced transactivation	0.1	(Ammosova et al. 2006)
MACS1-BR	18	1/6	R57G	Reduced TAR interaction and transactivation	1.5	(Delling et al. 1991)
MACS1-BR	22	1/6	K41E	K41 mutation reduces transactivation	0	(Kiernan et al. 1999; Rice and Carlotti 1990b)
MACS1-SPLN	28	1/4	S16G	S16 site of phosphorylation by CDK2, mutants associated with reduced transactivation	0.3	(Ammosova et al. 2006)
MACS2-BR	1,2	2/2	T64N	T64 site of phosphorylation by PKR, associated with reduced TAR binding	15	(Endo-Munoz et al. 2005)
MACS2-BR	2	1/2	K28R	K28 site of Tat acetylation by p300/PCAF, potentially decreased transactivation	0.3	(Kiernan et al. 1999)
MACS2-LN	34,35,36	3/3	T64N	T64 site of phosphorylation by PKR, associated with decreased TAR binding	15	(Endo-Munoz et al. 2005)
MACS3-BR	1,3,4,13,15	5/5	S62G	S62 site of phosphorylation by PKR, associated with decreased TAR interaction	3.7	(Endo-Munoz et al. 2005)
MACS3-BR	3	1/5	K51E	K51 site of acetylation by p300/PCAF, potentially decreased transactivation activity	0	(Kiernan et al. 1999)
MACS3-BR	13	1/5	Y47H	Y47H mutation associated with reduced transaction activity	1.7	(Kuppuswamy et al. 1989; Verhoef and Berkhout 1999)
MACS3-BR	15	1/5	R56Q	Reduced TAR interaction and transactivation	0.4	(Delling et al. 1991)
MACS3-LN	5,9,26,30	4/4	S62G	S62 site of phosphorylation by PKR, associated with reduced TAR interaction and transactivation activity	3.7	(Endo-Munoz et al. 2005)
MACS3-LN	5	1/4	T64D	T64 site of phosphorylation by PKR, associated with decreased TAR interaction and transactivation	2.8	(Endo-Munoz et al. 2005)
MACS3-LN	9,26,30	3/4	T64N	T64 site of phosphorylation by PKR, T64 mutants associated with reduced TAR binding	15	(Endo-Munoz et al. 2005)
MACS3-LN	9	1/4	C31R	C31 required for transactivation	0	(Rice and Carlotti 1990a)

The Los Alamos sequence database was searched using the search limits of Tat exon 1 and clade B HIV-1. Tat exon 1 sequences were selected from the search results, exported to align using CLC workbench. No additional selection criteria were utilised. The sequences represent a large selection of reported clade B Tat sequences (n=756). The alignment was used to determine the relative frequency of different amino acids at each position within the clade B Tat sequences. This was compared against the control HXB2 Tat sequence

and residue changes with a reported frequency of less than 20% in the Los Alamos HIV-1 sequence database. Sequence analyses revealed that a number of amino acid substitutions occurred in previously characterised and functionally conserved Tat domains and are predicted to directly impact the ability of Tat to transactivate the HIV-1 LTR. A Y-H mutation at position 47 (previously associated with reduced transactivation activity) was seen for subject CB1 in brain-, CSF- and PBMC-derived clones. Additionally, CSF-derived clones from this subject also harboured a mutation at positions 22 (C-Y) and 34 (C-R) also involved in the transactivation function of Tat (Kuppuswamy et al. 1989; Rice and Carlotti 1990a). Interestingly, the majority of Tat proteins isolated from subject CB1 had mutations associated with reduced transactivation function. Subjects CB3-PBMC (C34R), MACS1-BR (K41E), MACS3-BR (Y47H, R56Q) and MACS3-LN (C31R) also harboured amino acid substitutions directly impacting Tat's transactivation function. Amino acid substitutions were also identified in residues reported to be important in Tat post-translational modifications, including acetylation and phosphorylation, and possibly having indirect effects on Tat's transactivation functions. Mutations at the phosphorylation sites at amino acids 16, 46, 62 and 64 were observed in subjects MACS1-SPLN, MACS1-BR, MACS3-BR/MACS3-LN, CB3-CSF/CB3-SC/MACS2-BR/MACS2-LN/MACS3-LN, respectively.

Significantly, several amino acid changes identified have not been previously reported in the clade B sequence database including a unique C-R mutation at amino acids 31and 34 for subjects MACS3-LN and CB1-CSF/CB3-PBMC, respectively. Amino acid sequence changes with potential functional significance were identified in both CNS- and lymphoid-derived Tat sequences, reflecting heterogeneity in the patient Tat sequences.

Heterogeneity of CNS- and lymphoid-derived Tat-mediated LTR transactivation The major function of HIV-1 Tat is the regulation of viral transcription through interaction with TAR and subsequent transactivation of HIV-1 LTR (Dayton et al. 1986). The identification of nonconsensus sequence changes in conserved residues raises the potential for altered transactivation abilities of Tat derived from CNS- to lymphoid-tissue derived viruses. To assess the ability of Tat to transactivate the LTR, pTarget-Tat clones were co-transfected with a HXB2 LTR-Luciferase reporter into SVG glial and Jurkat T-cell lines. Semi-quantitative RT-PCR was used to show approximately equal levels of Tat, mRNA was observed for all Tat constructs analysed (data not shown). In both SVG and Jurkat cells, heterogeneity was observed in the ability of CNS- and lymphoid tissue-derived Tat to transactivate the LTR (Figs. 3 and 4, respectively). A subset of Tat clones from CNS- and lymphoid tissue-derived viruses of patients MACS1, MACS3, CB1 and CB3 had a restricted capacity to transactivate the LTR. This reflects sequence changes within functionally conserved Tat residues, particularly within the core domain (MACS1-BR22 K41E, MACS1-BR18 S46P), basic domain (MACS1-BR18 R57G), cysteine-rich domain (CB1-CSF26 C34R; CB1-CSF16 C22Y; CB3-PBMC26 C34R, MACS3-LN9 C31R) and the presence of an internal stop codon (CB1-PBMC3 W11*; Table 1). However, in each patient, no significant difference was identified in the mean ability of Tat from the CNS or lymphoid tissue compartments to transactivate the LTR, with each patient virus possessing multiple Tat variants that are capable of LTR transactivation. Furthermore, a pooled comparison of CNS and lymphoid Tat from all patients demonstrated a similar ability of Tat to transactivate the LTR. Thus, heterogeneity was observed in the ability of both CNS and lymphoid Tat to transactivate the LTR.

Discussion

In this study, we genetically and functionally characterised HIV-1 Tat from patients with HAD. HIV-1 Tat was successfully amplified, cloned and sequenced from viral isolates obtained from CNS to lymphoid tissue compartments from five HAD patients, generating a panel of 44 unique Tat clones. This represents a large panel of HIV-1 Tat variants cloned from patients with HAD and is a valuable resource for subsequent studies of the role of Tat in the pathogenesis of HAD.

Phylogenetic analysis revealed compartmentalisation of *tat* sequences between CNS- and lymphoid tissue-derived viruses in a subset of patients, MACS3 and CB3. Similar results have previously been described for *env* and *nef* sequences from MACS3 amplified directly from CNS to lymphoid tissues and matching virus isolates obtained via PBMC co-culture (Agopian et al. 2007; Thomas et al. 2007a, b). In contrast, *tat* sequences from MACS1, MACS2 to CB1, whilst clustering within distinct patient groups, failed to demonstrate sequence segregation between compartments. The lack of segregation of *tat* sequences between compartments may represent selection of viral variants during brain–PBMC co-culture, as has been previously reported for *nef* and *env* sequences amplified from primary isolates (Agopian et al. 2007; Thomas et al. 2007b). However, analysis of the *nef* and *env* sequences isolated from these same viruses does show distinct compartmentalisation (Gray et al., submitted and data not shown). This being the case, the biassed selection of particular *tat* variants via PBMC amplification of the virus isolates is unlikely and therefore, the tat sequences identified here are most likely representative of the

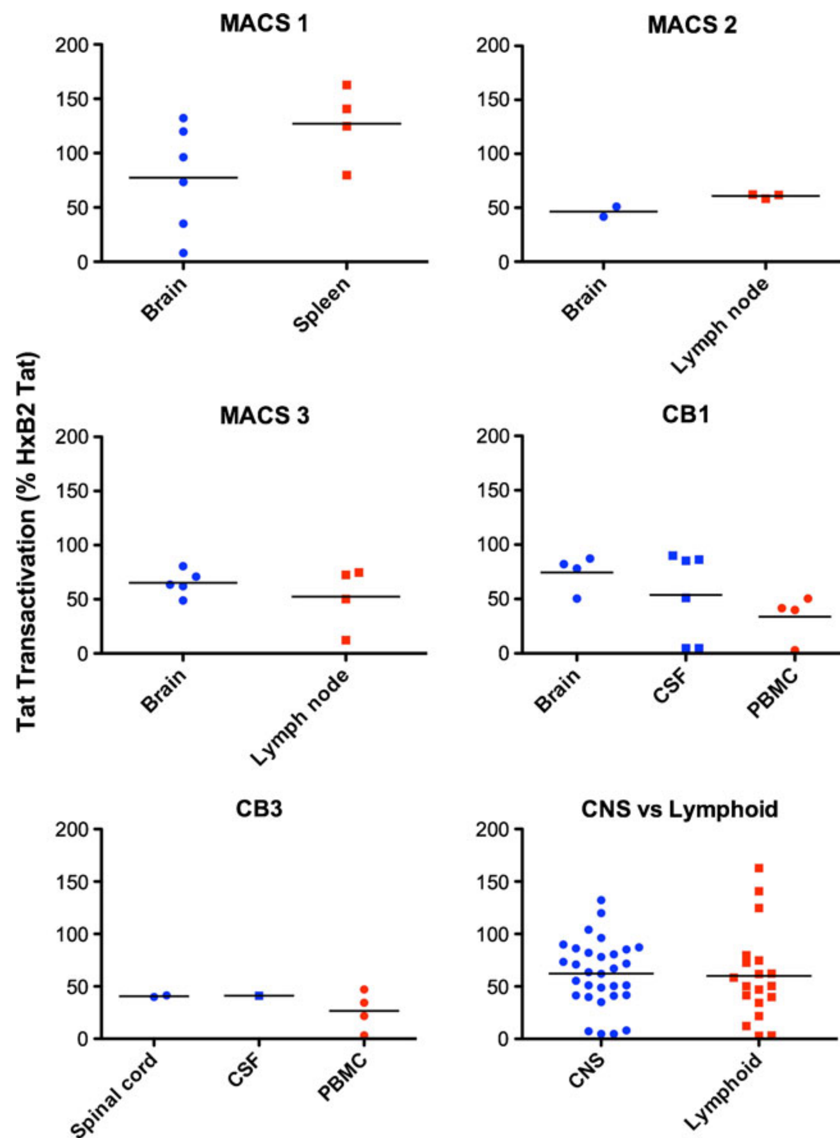

Fig. 3 Tat LTR transactivation of CNS and lymphoid Tat in SVG astrocytes. Tat clones from CNS- to lymphoid-derived viruses from patients MACS1, MACS2, MACS3, CB1 to CB3 were co-transfected with a LTR luciferase reporter into SVG astrocyte cells. CNS-derived Tat are shown in *blue*, lymphoid-derived Tat in *red*. Tat transactivation is plotted as a % HXB2 Tat LTR transactivation. *Bar* represents mean for the data set. Data is representative of two experiments performed in triplicate

replicating viruses present within the CNS and lymphoid tissue compartments. Nonetheless, additional studies isolating *tat* RNA directly from CNS to lymphoid tissues from HAD patients could be conducted to reduce potential bias.

Analysis of Tat amino acid sequences revealed heterogeneity in both CNS- and lymphoid-derived Tat variants, with a number of amino acid changes occurring in residues previously reported to be conserved in clade B Tat sequences. Furthermore, multiple amino acid substitutions were identified in each patient that segregated CNS and lymphoid tissue Tat. Molecular diversity of the HIV-1 genome is a key regulator of viral replication and fitness (Andreoni 2004). HIV-1 Tat demonstrates significant variation in sequence, which is associated with altered capacity for transactivation of the LTR (Sivakumaran et al. 2007; Yukl et al. 2009). Furthermore, the brain is an immunologically privileged site with reduced selection pressure and constraints on viral evolution potentially impacting the molecular evolution of Tat. In agreement with our study, previous studies have shown heterogeneity among brain-derived Tat sequences, with molecular heterogeneity greatest among patients with HAD (Bratanich et al. 1998; Mayne et al. 1998). Here, we also demonstrate within HAD patients, heterogeneity in the ability of CNS- and lymphoid-derived Tats to transactivate the HIV-1 LTR. However, we did not identify significant differences between the ability of CNS- and lymphoid-derived Tats to transactivate the LTR, either within patients or when

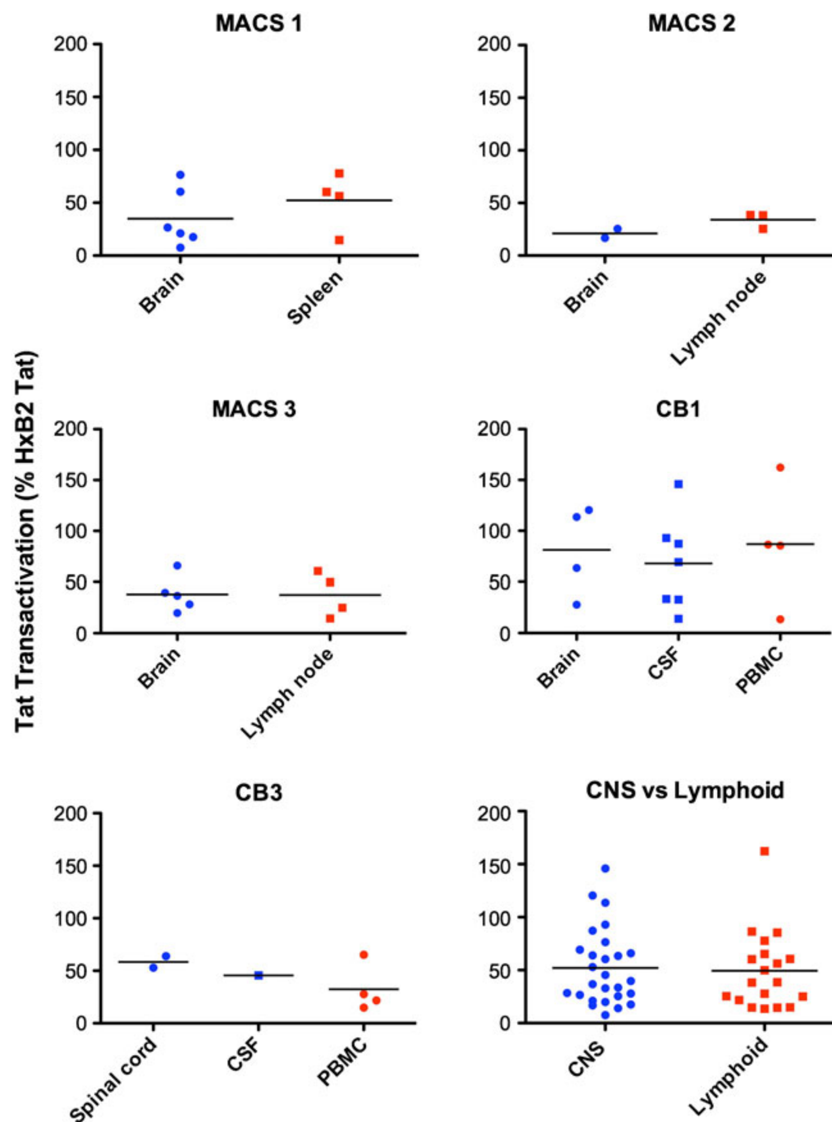

Fig. 4 Tat LTR transactivation of CNS and lymphoid Tat in Jurkat T cells. Tat clones from CNS to lymphoid derived viruses from patients MACS1, MACS2, MACS3, CB1 to CB3 were co-transfected with a LTR luciferase reporter into Jurkat T cells. CNS derived Tat are shown in *blue*, lymphoid derived Tat in *red*. Tat transactivation is plotted as a % HXB2 Tat LTR transactivation. *Bar* represents mean for the data set. Data is representative of two experiments performed in triplicate

comparing Tat from all patients. A previous cross-sectional study directly amplifying Tat from CNS tissues reported that Tat from HAD patients had impaired LTR transactivation capacity when compared to non-HAD patients (Boven et al. 2007). However, our results demonstrate that the majority of Tat clones from CNS-derived viruses in each patient have the capacity to transactivate the LTR. Thus, our data indicates the maintenance of Tat with functional LTR transactivation in the CNS of HAD patients.

A subset of Tat clones from both CNS and lymphoid tissue compartments was unable to transactivate the LTR. The loss of activity correlated with amino acid substitutions of residues within the cysteine-rich, core and basic domains and previous functional studies on these domains; although further mutagenesis studies on the Tat clones in this study are required to definitively identify the residues involved. Previous analyses of Tat sequences from HAD patients have also identified functionally defective Tat (Boven et al. 2007; Thomas et al. 2007a). The significance of these defective Tat variants in the context of other functional variants remains unclear. Tat mutants with defective LTR transactivation due to substitution of residues in the cysteine and basic domains have been shown to act as transdominant-negative mutants, inhibiting its wild-type counterpart either by sequestration of cellular co-factors or by binding to TAR without initiating transactivation (Balboni et al. 1993; Caputo et al. 1996; Meredith et al. 2009; Orsini and Debouck 1996). Tat can be released via a

leaderless secretory pathway from HIV-1-infected cells to the extracellular space and subsequently taken up by uninfected cells (Chang et al. 1997; Ensoli et al. 1993; Liu et al. 2000). In addition, Tat exploits neuronal transport mechanisms to facilitate the passage of Tat through the CNS, allowing the biological effects of Tat to occur at sites distant from its production (Bruce-Keller et al. 2003; Chauhan et al. 2003). This raises the possibility that defective Tat variants identified in this study may act to modulate or inhibit HIV-1 LTR transcription *in vivo* by acting via a transdominant-negative mechanism at sites distant from the site of production. Furthermore, it is possible that the viral isolates used in this study, representing replication competent virus and not amplified from tissue viral RNA, may under represent the degree of defective Tat variants with the CNS, consistent with previous reports (Thomas et al. 2007a). Additional studies are required to determine the significance of defective Tat variants in modulating LTR transactivation in the CNS.

HIV-1 Tat acts as a potent neurotoxin (Eugenin et al. 2007; Mishra et al. 2008; Nath et al. 1996) and chemo-attractant (Albini et al. 1998), and is capable of inducing and modulating the expression of a variety of host genes. The neurotoxic and chemo-attractant domains map to regions spanning amino acids 31–61 and 24–51, respectively. Here, we identified amino acid sequence substitutions in the neurotoxic and chemotactic domains that segregated CNS- and lymphoid-derived Tat. The majority of studies on the neurotoxic and chemotactic properties have focused on clade B consensus Tat which is not representative of Tat proteins in the CNS. Furthermore, amino acid substitutions within these domains outside the clade B consensus affect both properties (Mishra et al. 2008; Ranga et al. 2004). Thus, our data raises the potential for the evolution of unique Tat variants in the CNS with altered neurotoxic and chemotactic proprieties. Such CNS variants may have an impact on HAD pathogenesis. In this context, additional studies are currently underway to further characterise the neurotoxic and chemotactic capacity of the CNS- and lymphoid-derived Tat identified here.

Materials and methods

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

Plasmids Plasmids containing the HIV-1 proviruses NL4-3, AD8, JRCSF and YU2 have been constructed as previously described (Adachi et al. 1986; Collman et al. 1992; Koyanagi et al. 1987; Li et al. 1991).

Cell lines SVG astrocytes (Major et al. 1985) were maintained in minimum essential medium (Invitrogen) supplemented with Earle's salts, 20% (vol/vol) foetal calf serum, 100 µg penicillin and streptomycin per ml. Jurkat T-cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% (vol/vol) foetal calf serum, 100 µg penicillin and streptomycin per milliliter. All cells were maintained in a humidified 37°C incubator supplemented with 5% CO_2.

Tat cloning, sequencing and phylogenetic analysis Viral RNA was purified from HIV-1 isolates using a QIAMP UltraSens Virus purification kit (Qiagen) according to the manufacturer's protocol. cDNA was generated using SuperScript III reverse transcriptase (Invitrogen) and random hexamers according to the manufacturer's protocol. A 255-bp fragment spanning the first exon of HIV-1 *tat* (corresponding to nucleotides 5809–6064 of HIV-1 NL4.3) was amplified using nested primers and high fidelity Taq polymerase and primers; outer-TatF1 (5′-CATTTCAGAATTGGG-3′) and -TatR1 (5′-TTGCTATTATTATTGCTAC-3′), inner-TatF3 (5′-ACTC GACAGAGGAGAGCAAG-3′) and -TatR3 (5′-GCATTA CATGTACTACTTACTGC-3′). PCR cycling conditions consisted of an initial denaturation step of 94°C for 5 min followed by 30 cycles of 94°C for 15 s, 50°C for 30 s and 72°C for 30 s, followed by a final extension of 72°C for 7 min. Multiple, independent amplified products were pooled and purified using High Pure PCR product purification kit (Roche) according to the manufacturer's protocol. PCR products were inserted into the pTargeT expression plasmid (Promega, WI) by TA cloning. For the generation of reference Tat clones, the proviral plasmids of HIV-1 HXB2, NL4-3, AD8, JRCSF and YU2 were used as template DNA for PCR, and amplified products were similarly inserted into pTargeT. The *tat* coding region was sequenced by using T7 forward primer with Big Dye terminator V3.1 sequencing chemistry (Applied Biosystems) on a Applied Biosystems 3730×1 DNA sequencer. Sequence alignments were generated using CLC workbench (CLC Bio). Neighbour-joining phylogenetic trees, bootstrapped 100 times were produced using CLC workbench (CLC Bio). The relative frequency of amino acid substitutions were compared to published clade B Tat sequences deposited in the Los Almos HIV-1 Sequence Database (http://www.hiv.lanl.gov/).

Tat Transactivation assay SVG cells were transfected using 0.25 µl of Lipofectamine 2000 (Invitrogen) at approximately 60% confluence in a 96-well format with 180 ng HIV-1 HxB2 LTR luciferase reporter and 5 ng of pTarget-Tat that expresses HIV-1 Tat 72 under the control of the CMV promoter. Jurkat cells (7.5×10^6) were transfected with 5.5 µg of HIV-1 HxB2 LTR luciferase reporter and 2 µg pTarget-Tat via electroporation using BIORAD Gene Pulser

II with capacitance extender (BIORAD). Electroporation conditions used were a voltage of 250 V, capacitance of 960 μF, resistance 200 Ω. Transfection efficiency was monitored using an eGFP (Clontech) control vector. At 48 h, posttransfection cells were harvested and assayed for luciferase according the manufacturer's protocol (Promega). Luciferase values were normalised to protein concentration in cell lysates using reducing agent compatible BCA protein assay (Pierce). Fold LTR transactivation was normalised to HxB2 Tat72 which was used as a positive control.

Nucleotide sequence accession numbers The *tat* nucleotide sequences reported here have been assigned GenBank accession numbers HQ174416 to HQ174459.

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