HIV-1 Clade-Specific Differences in the Induction of Neuropathogenesis

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Human immunodeficiency virus (HIV)-associated dementia (HAD) is common among clade B HIV-infected individuals, but less common and less severe among individuals infected with clade C HIV-1, suggesting clade-specific differences in neuropathogenicity. Although differences in neuropathogenicity have been investigated *in vitro* using viral proteins responsible for HAD, to date there are no virological studies using animal models to address this issue. Therefore, we investigated neuropathogenesis induced by HIV-1 clades using the severe combined immune deficiency (SCID) mouse HIV encephalitis model, which involves intracranial injection of macrophages infected with representative clade B (HIV-1_{ADA}) or clade C (HIV-1_{Indie-CI}) HIV-1 isolates into SCID mice. In cognitive tests, mice exposed to similar inputs of HIV-1 clade C made fewer memory errors than those exposed to HIV-1 clade B. Histopathological analysis of mice exposed to clade B exhibited greater astrogliosis and increased loss of neuronal network integrity. *In vitro* experiments revealed differences in a key characteristic of HIV-1 that influences HAD, increased monocyte infiltration. HIV-1_{Indie-CI}-infected macrophages recruited monocytes poorly *in vitro* compared with HIV-1_{ADA}-infected macrophages. Monocyte recruitment was HIV-1 Tat and CCL2 dependent. This is the first demonstration, ever since HIV neuropathogenesis was first recognized, that viral genetic differences between clades can affect disease severity and that such studies help identify key players in neuropathogenesis by HIV-1.

Key words: dementia; human; virus; cognitive; lentiviruses; macrophage

Introduction

Human immunodeficiency virus (HIV)-associated dementia (HAD) affected 15–30% of infected individuals before the widespread use of highly active antiretroviral therapy (HAART) in the United States (McArthur et al., 1993). Despite reduced HAD incidence with the advent of HAART (Sacktor et al., 2001; Dore et al., 2003), improved survival rates of HIV-infected individuals have led to increase in cognitive impairment resulting from chronic CNS HIV exposure (Sacktor et al., 2001; Langford et al., 2003). In contrast, incidence/prevalence of severe forms of HAD is reported to be considerably lower (2–4%) in the HAART-naïve HIV-1-infected population in India, although milder forms of

disease have been reported (Satishchandra et al., 2000; Riedel et al., 2006; Gupta et al., 2007). The Indian acquired immunodeficiency syndrome (AIDS) epidemic is dominated by clade C HIV-1 (Chakrabarti et al., 2000; Siddappa et al., 2004) and the clade C HIV-1 is found in brain autopsies (Mahadevan et al., 2007) from HIV-infected Indian patients suggesting a clade-specific difference in the neuropathogenesis of HIV-1. Clade C HIV-1 is responsible for more than half of new HIV-1 infections worldwide (Geretti, 2006) and a major proportion of HIV infections in India (Siddappa et al., 2004), whereas in North America, HIV-1 clade B is responsible for nearly all HIV-1 infections.

Mechanisms underlying the pathogenesis of HAD, characterized by neuronal injury, are complex, including infiltration of monocyte-derived macrophages (MDMs) into brain and neurotoxic effects of HIV-1 proteins (e.g., gp120 and Tat). Tat plays multiple roles in HAD pathogenesis, including direct neurotoxicity (Gourdou et al., 1990; Sabatier et al., 1991), chemokine dysregulation (Conant et al., 1998; Weiss et al., 1999), and cytokine induction. A dicysteine motif in Tat has been implicated in direct monocyte chemotaxis *in vitro* (Albini et al., 1998). We previously reported that clade C Tat is divergent from other HIV-1 clades in that the dicysteine motif (C30C31), which is highly conserved in all clades examined, exhibits a C31S polymorphism (Ranga et al., 2004), rendering it defective for monocyte chemotaxis *in vitro*.

Differential distribution of B and C HIV-1 clades and differences in host genetics and access to antiretrovirals make it diffi-

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cult to assess neuropathologic differences between populations infected by B and C HIV-1 clades. These difficulties are largely overcome by the use of the severe combined immune deficiency mouse HIV encephalitis (SCID HIVE) model (Tyor et al., 1993; Persidsky et al., 1996), in which for the first time the two HIV-1 clades are compared in a single host. This model involves intracranial injection of MDMs infected by HIV-1 into SCID mice, and its strength lies in its ability to recapitulate key pathological and behavioral features of human HAD (Avgeropoulos et al., 1998; Cook et al., 2005). Importantly, these mice also exhibit behavioral deficits when tested in a water radial arm maze (WRAM) (Avgeropoulos et al., 1998; Sas et al., 2007), which are consistent with cognitive dysfunction in human HAD. In this report, we primarily investigate neuropathogenic differences of clade B and clade C HIV-1 isolates. We find that neuropathogenesis by a clade C HIV-1 results in a milder cognitive dysfunction than with a clade B HIV-1. We also show that (1) HIV-infected MDMs or the media from such cells can recruit monocytes; (2) that monocyte recruitment is Tat and CCL2 dependent; and (3) that clade C HIV-1 is significantly compromised for monocyte chemotaxis. Our work shows that both direct and indirect effects of HIV-1 Tat protein are compromised in subtype C HIV-1 in a virological setting.

Materials and Methods

HIV-1 infection, animals, and behavioral testing. Primary human MDMs (purchased from the University of Nebraska Medical Center) were cultured in DMEM (Invitrogen) with 10% human serum (Sigma), penicillin-streptomycin (Invitrogen), and macrophage colony-stimulating factor (M-CSF; Sigma) at 6.6 ng/ml at 37°C with 5% CO₂ for 7 d in Teflon-coated flasks to prevent attachment. To ensure that the HIV-infected MDMs used for intracranial injection had equivalent viral loads, $\sim 5 \times 10^6$ MDMs were infected with HIV-1_{ADA} (clade B) at a multiplicity of infection (MOI) of 0.1 (1 h) or HIV-1_{Indie-C1} (clade C) at an MOI of 0.01 (3 h). HIV-1_{ADA} was obtained from the University of Nebraska Medical Center, and HIV-1_{Indie-C1} was prepared by transfecting 293T cells with p93IN101 DNA (Mochizuki et al., 1999). Uninfected MDMs were used as controls. After infection, MDMs were resuspended in medium devoid of M-CSF and cultured for 14 d with media changes every third day. At the end of 14 d incubation, both sets of MDMs displayed similar viral load as determined by immunocytochemistry with anti-p24 antibodies (DAKO) and ELISA to measure p24 levels in the media (PerkinElmer Life and Analytical Sciences) (see Fig. 2). MDMs, grown in Teflon-coated flasks, are recovered by centrifugation and resuspended in PBS for inoculation into mice.

Eighteen 4-week-old C57BL SCID mice (Jackson Laboratory) were acclimated to the animal room for 1 week before injection. Mice were single-housed in microisolator cages (biosafety level-3 equivalent). The animal room was set on a 12 h light cycle. Cages, bedding, food, and water were sterilized before use. Animal protocols were approved by the Medical University of South Carolina's Institutional Animal Care and Use Committee. Approximately 1×10^5 HIV-1-infected MDMs (clade B or C) or uninfected MDMs were injected into the right frontal lobe of 5-week-old SCID mice ($n = 6$ per group). Six days after intracranial injections, WRAM behavior testing ensued. This win-shift WRAM uses water escape onto hidden platforms as the reinforcer (Hyde et al., 1998; Bimonte-Nelson et al., 2003; Hunter et al., 2004). The eight-arm maze had submerged escape platforms placed on the ends of four arms. Each subject had different platform locations that remained fixed throughout the experiment. A subject was released from the start arm and had 2 min to locate a platform. Once a platform was found, the mouse remained on it for 15 s, and was then returned to its heated cage for 30 s until the next trial. During the interval, the platform found by the mouse was removed from the maze. The animal was then placed again into the start alley and allowed to locate a second platform. The same sequence of events was repeated daily until all four platforms were located. Thus, for each animal, a daily session consisted of four trials, with the number of plat-

formed arms reduced by one on each subsequent trial, resulting in the working memory system becoming increasingly demanding as trials progressed. Animals received 4 trials/d for 12 d. The testing protocol included an initial learning phase (days 1–8) and a final testing phase (days 9–12). Working memory correct (WMC) and working memory incorrect (WMI) errors were quantified based on the orthogonal measures of Jarrard and others (Jarrard, 1993; Hyde et al., 1998; Bimonte-Nelson et al., 2003; Hunter et al., 2004). WMC errors were defined as entries into an arm wherein a platform had already been located, and WMI errors were defined as repeat entries into an arm that never contained a platform.

Immunohistochemistry, densitometry, and detection of HIV RNA levels using real-time PCR. After WRAM testing, mice were killed and brains extracted and frozen for immunohistochemistry ($n = 6$). Approximately 13 sets of serial 5 μ m, coronal sections were taken from the frontal through the temporoparietal lobes of each mouse. Each set was separated by 46 intervening 5 μ m sections, which were saved for RNA extraction. Each stained set consisted of slides that underwent immunoperoxidase staining separately for human macrophages (EBM-11; Dako); HIV-1 p24 (p24; DAKO); astrocytes (GFAP; Millipore Bioscience Research Reagents) and neuronal dendrites (MAP2; Millipore Bioscience Research Reagents) as described previously (Avgeropoulos et al., 1998; Sas et al., 2007). Average numbers of primary human macrophages (EBM-11-positive cells) and HIV-infected cells (p24 positive cells) were determined by light microscopy. Immunoperoxidase-stained slides were imaged at 20 $\times$ using an Olympus microscope. Densitometry analysis was performed to determine the intensity of immunoperoxidase stain using NIH Image software on the images (20 $\times$) representing sections stained for astrogliosis (GFAP) and neuronal network integrity (MAP2) (Sas et al., 2007). For measuring neuronal integrity (MAP2), the left hemisphere (non-injected hemisphere) of each mouse served as a normal control and was set at 100%. The measured value of the right hemisphere was compared with the left hemisphere of the section to determine the relative differences in MAP2 staining. Three sections per mouse from six mice per group were analyzed in the above manner to derive the final values plotted in Figure 3.

For real-time PCR measurements of viral RNA, the tissue from these intervening sections was homogenized, and the RNA was extracted according to the RNeasy (Qiagen) protocol. Approximately 1 μ g of total RNA was used for cDNA synthesis. First-strand cDNA synthesis was performed using the High Capacity cDNA Archive kit (Applied Biosystems). HIV-1 gag RNA levels were determined using real-time PCR with ABI 7000 Prism (Applied Biosystems) to determine viral load, according to the manufacturer's suggested protocol.

Measurement of monocyte migration using a dual-chamber assay. Human MDMs were cultured for 4 d under the same conditions as described above. Approximately 2×10^5 MDMs were plated in the lower wells of a 24-well plate (Rao et al., 2009). Approximately 2×10^5 cultured MDMs were infected with HIV-1_{ADA} virus (clade B) at an MOI of 0.1 for 1 h or HIV-1_{Indie-C1} (clade C) at an MOI of 0.01 for 3 h and incubated for 5 d in the above-mentioned medium devoid of M-CSF. ELISA for p24 in the supernatant staining and p24 staining of macrophages were used to ensure equal viral load for both HIV-1_{ADA} and HIV-1_{Indie-C1}. In the upper chamber, 2×10^5 elutriated human monocytes were added. To facilitate their identification subsequent to migration, monocytes were stained with carboxyfluorescein succinimidyl ester (CFSE) dye before adding them to the transwells. After 24 h of incubation, cells in the lower chamber were examined in a fluorescence microscope to quantify the migrated cells.

Neutralization of Tat and CCL2 proteins. Neutralization of Tat and CCL2 proteins was performed by a slightly modified version of the earlier technique (Weiss et al., 1999). Four days after HIV-1 infection, the infected MDMs, prepared the same way as described in the migration experiments, were incubated with fresh medium (DMEM 2% human serum) for 24 h (Rao et al., 2009). The conditioned medium (supernatant from HIV-1-infected and control wells) was then incubated for 1 h with Pansorbin beads (EMD Biosciences) to which either anti-Tat [E1.1, which neutralizes both Tat proteins (U. Ranga, unpublished observations)] or anti-CCL2 antibodies (MAb 279; R&D Systems) were pre-bound. Optimal antibody concentrations were determined for Tat by testing increasing concentrations of antibodies and for CCL2 based on the CCL2 levels in the medium determined by ELISA. Beads were re-

moved by pelleting, and the supernatant was used in migration experiments following the above-mentioned migration protocol.

Tat-induced and HIV infection-induced secretion of CCL2 from MDMs and astrocytes. Four days after HIV infection, MDMs were incubated for 24 h in fresh medium without human serum to eliminate the contribution of CCL2 in human serum. The day 5 no-serum supernatant was collected, and ELISA for CCL2 (R&D Systems) was performed in triplicates in three separate experiments as described previously (Eugenin et al., 2006).

Statistical analysis. Statistical analysis for the behavior study was via StatView. Repeated-measures ANOVA analysis was performed, and the *p* values were obtained by comparing errors made by clade B, clade C, and control mice individually with each other. For migration studies, chemokine expression, and histopathology analysis, the significance and *p* values were determined using Microsoft Excel.

Results

SCID mice with clade C HIV-1 display milder cognitive deficits than clade B HIV SCID mice

The SCID mouse HIV encephalitis model, developed originally by Tyor et al. (1993) and subsequently improved by both W. Tyor and coworkers and H. Gendelman and coworkers, is a useful, sensitive, and well characterized animal model to test HIV-induced neurotoxicity and behavioral changes (Persidsky et al., 1996, 1997; Avgeropoulos et al., 1998; Cook et al., 2005; Sas et al., 2007). Behavioral testing with WRAM revealed that clade B HIV mice exhibited poor working memory as shown on two orthogonal measures of working memory competence. Clade B HIV mice made more WMI (a form of long-term memory) and WMC (a form of short-term memory) errors when compared with controls (Fig. 1). Repeated-measures ANOVA to analyze the number of errors, which were collapsed across testing days (days 9–12) and trials, revealed that clade B HIV mice made significantly more WMI errors than clade C HIV mice ($p = 0.046$) (Fig. 1*a*) or the controls ($p = 0.005$) (Fig. 1*a*) injected with uninfected MDMs. Clade B HIV mice made significantly more WMC errors than the controls ($p = 0.042$) (Fig. 1*b*). Clade C HIV mice made an intermediate number of WMI errors between clade B and the controls (Fig. 1*b*). Moreover, clade B HIV mice were less successful in handling an increasing working memory load as shown on two dependent variables. Indeed, clade B HIV mice made significantly more WMC and WMI errors than clade C HIV or the control mice as the memory load increased during the later trials (Fig. 1*c,d*).

Brains of SCID mice exposed to either HIV-1 clade B or HIV-1 clade C show similar viral loads, but those with clade C HIV-1 show decreased pathology

Evaluating different clades of HIV-1 for their potential to induce SCID HIV requires that the viral load of these related, but distinct, viruses are comparable. Therefore, in addition to ensuring that the MDMs injected into mouse brain contained equal proportions of HIV-positive cells with similar viral replication (Fig. 2), immunohistochemical analysis of the frozen sections was per-

Figure 1. Cognitive performance of HIV-infected and control SCID mice in the latter phase of testing. *a, b*, Cognitive performance of HIV-infected and control mice averaged across days (9–12) and trials [1–4 trials for WMI (a form of long-term memory; *a*) and 2–4 trials for WMC (a form of short-term memory; *b*)]. Clade B SCID mice made significantly more WMI errors than clade C ($p = 0.046$) or controls ($p = 0.005$). Clade B SCID mice made significantly more WMC errors ($p = 0.042$) than controls. Clade C mice made an intermediate number of errors for two orthogonal working memory measures, which suggests that memory impairment induced by clade C HIV-1 virus is less profound than impairment induced by clade B HIV-1. *c, d*, Mean WMI and WMC errors, respectively, for each trial for the latter phase of testing. Clade B HIV mice were less successful at handling increasing working memory load with two dependent variables. t1–t4, Trials 1–4.

formed with antibodies to HIV-1 p24 (Fig. 2). This analysis showed equal numbers of HIV-positive cells in the brains of SCID mice infected with either virus [11 ± 4 vs 13 ± 5 (mean $\pm$ SD) p24-positive cells per section for clade B and clade C HIV-infected SCID mouse brains, respectively; the data represent an average of five sections per mouse brain around the injection site in the frontal cortex region and nine mice in each group]. Quantitative reverse transcription-PCR analysis of HIV-1 clade B- and clade C-infected mouse brains ($n = 6$; from frontal cortex) revealed average log copy numbers of 12.75 ± 0.11 and 12.36 ± 0.16 (mean $\pm$ SEM), respectively (Fig. 2).

Astrogliosis, as evidenced by GFAP staining, and a decrease in neuronal network integrity, as indicated by reduction in MAP2 staining, were observed in the brains of mice injected with HIV-infected MDMs in frontal lobe sections around the injection tract. Brains of control mice also displayed low levels of astrogliosis and a slight reduction in MAP2 staining, suggesting background inflammation. However, the severity of astrogliosis in the frontal cortex region was greatest in clade B HIV-infected SCID mice (Fig. 3*a*) compared with clade C HIV ($p = 0.0117$) and control ($p = 0.0008$) mice. Percentage MAP2 staining comparing the right and the left frontal cortex regions for neuronal integrity revealed a significant difference between brains of mice injected with clade B HIV-1 and control brains ($p = 0.0006$) but no statistically significant differences between the two HIV-1 clades (Fig. 3*b*). Nevertheless, a trend for less severe MAP2 changes was observed in clade C HIV mice compared with clade B HIV-1 HIV mice.

Figure 2. *In vitro* and *in vivo* quantitation of viral infectivity in injected MDMs and infected mouse brains. *a*, HIV-1 p24 immunostaining of clade B and clade C HIV-infected MDMs in culture before injecting into mouse brain. The p24-positive cells are stained dark to medium blue (shown by black arrows). *b*, p24 immunostaining of frontal cortex sections of clade B and clade C HIV-infected mouse brains along the injection track 17 d after injection. Arrows point to some of the p24-positive cells. *c*, Quantitation of HIV-infected cell numbers from *a* and *b* as well as the viral RNA copy numbers in frontal cortex of mouse brains as determined by real-time PCR with clade-specific primers and probes. The results show that the viral load for each clade of HIV-1 is similar both in the infected MDMs before injection and 17 d after injection in the brains of mice.

Figure 3. Quantitative assessment of immunoperoxidase-stained sections of HIV-infected and control mouse brains. *a*, Measurement of GFAP-positive cells to determine astroglia. Densitometric analysis of five frontal cortex GFAP-stained sections from the brains of six mice in each group injected with uninfected, clade B HIV-infected, and clade C HIV-infected MDMs at 17 d after injection. *b*, Measurement of MAP2-positive cells to examine neuronal network integrity [from 3 sections per mouse ($n = 6$) around the site of injection in the frontal cortex and comparing the right hemisphere to the left] at 17 d after injection. Clade C HIV mice show distinctly lower astroglia than clade B HIV mice, while showing an intermediate loss of MAP2 stain between clade B HIV mice and control mice. Error bars represent SEM.

Clade C HIV-infected MDMs display reduced monocyte chemotaxis

Monocyte infiltration into the brain is a hallmark of HAD. Although Tat causes monocyte chemotaxis *in vitro*, monocyte chemotaxis by Tat in the context of HIV-infected cells has not been demonstrated. To investigate clade differences in monocyte chemotaxis, we used MDMs, HIV-1 susceptible cells that are relevant to HIV-1 neuropathogenesis, that were infected with either HIV-1_{ADA} (clade B) or HIV-1_{Indie-C1} (clade C). HIV-infected MDMs

were added to the bottom chamber of the transwell system, and elutriation-derived, undifferentiated human monocytes were added to the top chamber (Rao et al., 2009). After 24 h of incubation, we observed that clade B-infected MDMs attracted 64% ($p = 3.5 \times 10^{-5}$) more monocytes than did clade C-infected MDMs (Fig. 4*a*). Supernatant p24 ELISA (PerkinElmer Life and Analytical Sciences) measurements were done before the migration experiments to ensure that the two sets of MDM cells had similar viral loads.

Chemotaxis induced by HIV-infected MDMs is mediated by Tat and CCL2

HIV-infected MDMs secrete a number of soluble factors into the medium, including Tat protein. To delineate the contribution of factors influencing monocyte chemotaxis, the medium from infected MDM cell culture was first confirmed to induce chemotaxis using the *in vitro* dual-chamber assay described earlier (Fig. 4*b*, no treatment). Then, to determine whether extracellular Tat plays a role in chemotaxis, the medium was immunodepleted using anti-Tat neutralizing antibodies. These experiments demonstrated that a greater proportion of monocytes recruited by clade B HIV-infected MDMs were attributable to Tat compared with clade C HIV-infected MDMs. Similarly, immunodepletion of CCL2 revealed that CCL2 also plays a key role in monocyte chemotaxis caused by infection by either clade of HIV-1 (Fig. 4*b*).

Poor induction of CCL2 chemokine by clade C HIV-1 and its Tat protein

Tat released from infected cells can gain access into uninfected cells in the vicinity (Schwarze et al., 1999). A transcriptional transactivator of both viral and cellular genes, Tat induces astrocytes and monocytes *in vitro* to produce chemokines (e.g., CCL2) (Conant et al., 1998; Weiss et al., 1999; D'Aversa et al., 2004). Therefore, we investigated the mechanistic basis for lowered monocyte chemotaxis by clade C Tat protein. We measured the production of CCL2 by monocytes and astrocytes after the addition of Tat protein from either clade to cultures for 24 h. Clade C Tat protein, compared with clade B Tat, induced significantly lower levels of CCL2, in a dose-dependent manner, in both MDMs (100 ng of Tat, $p = 1.4 \times 10^{-5}$; 500 ng of Tat, $p = 6.1 \times 10^{-10}$) and astrocytes (100 ng of Tat, $p = 0.0017$; 500 ng of Tat, $p = 0.0001$) (Fig. 5*a,b*).

To test the relevance of this finding in the context of HIV-1 replication, we measured the secretion of CCL2 by clade B and C HIV-1-infected MDMs. HIV-infected MDMs were incubated for 24 h in serum-free medium to eliminate the confounding presence of CCL2 in human serum used for growing MDMs. Clade C HIV-infected MDMs displayed a significantly reduced ability to secrete CCL2 in comparison with clade B-HIV-infected MDMs ($p = 0.0124$) (Fig. 5*c*).

Figure 4. Monocyte migration assays in the presence of HIV-1-infected MDMs using a dual-chamber assay. **a**, Monocyte migration caused by clade B and clade C HIV-infected MDMs. The numbers plotted reflect the monocytes detected in the lower chamber by counting the CFSE-positive cells. Numbers plotted represent means from three experiments each (2 quadruplicates and 1 triplicate) ($n = 11$). **b**, Monocyte migration caused by spent medium from HIV-infected MDMs can be blocked by anti-Tat or anti-CCL2 antibodies. The numbers represent fluorescent cells detected in the lower chamber after 24 h of incubation. Bars labeled " α -Tat Ab" and " α -CCL2 Ab" represent experiments in which spent media from HIV-infected MDMs were first treated with antibodies as described in Materials and Methods. Numbers plotted represent means from a pentuplicate experiment ($n = 5$). Clade C HIV-infected cells recruit significantly less monocytes than clade B HIV-infected cells, and the monocyte recruitment by the medium from both types of cell is mediated by Tat and CCL2. Error bars represent SEM.

Figure 5. Differential induction of CCL2 by HIV-infected and Tat-treated MDMs and astrocytes (*in vitro*). **a**, Measurement of CCL2 secreted by MDMs exposed to Tat protein from clade B or clade C HIV-1 at 100 and 500 ng/ml. Control represents cells incubated with untreated medium ($n = 6$). **b**, Measurement of CCL2 secreted by human astrocytes exposed to Tat protein from clade B or clade C HIV-1 at 100 and 500 ng/ml. Control represents cells incubated with untreated medium ($n = 8$). **c**, Measurement of CCL2 in the media of MDM-infected with clade B or C HIV-1. Control represents uninfected MDMs ($n = 6$). Both Tat treatment and HIV infection of primary MDMs and astrocytes show that clade C HIV-1 is compromised for the induction of CCL2 secretion. Error bars represent SEM.

Discussion

Reports of reduced prevalence of severe forms HAD in regions with predominantly clade C HIV infections, such as India (Satishchandra et al., 2000; Riedel et al., 2006; Gupta et al., 2007), imply clade-specific differences in neuropathogenesis. To verify this hypothesis, we have used *in vitro* experiments with purified Tat proteins and *in vivo* experiments using infectious molecular clones of HIV-1 in the SCID HIVE model. We have extended our previous findings that clade C Tat is defective in its chemotactic properties. Our results demonstrate, for the first time, differences in neuropathogenesis between clade B and C HIV-1 isolates in an *in vivo* model. Differential neuropathogenesis observed for the

two clades *in vivo* also correlated with differential ability of HIV-infected MDMs to recruit monocytes *in vitro* and in inducing chemokines such as CCL2 in infected cells.

Our results revealed significant differences between the two clades in terms of behavioral deficits. Clade B HIVE mice made the most working memory errors and clade C HIVE mice made an intermediate number of errors in reference to clade B and control mice. Our approach of injecting HIV-infected macrophages into the frontal cortex produces well documented neuropathological and behavioral effects that are widespread rather than limited to the site of injection or right side alone. As described previously (Griffin et al., 2004), multiple areas of the brain are likely involved in causing the behavioral abnormalities, including frontal lobes, hippocampus, and basal ganglia. Some of these effects require a relatively remote action of HIV. These effects are thought to be related to diffusible substances produced by HIV-infected macrophages and activated glia (Griffin et al., 2004). Our behavioral testing protocol does not include WRAM testing of uninoculated normal SCID mice, because they have been previously shown (Avgeropoulos et al., 1998) to perform consistently with, or even better than, our controls injected with uninfected MDMs.

MAP2 staining serves both as a means of measuring HIV-induced damage to neuronal network integrity (Avgeropoulos et al., 1998; Zheng et al., 2001; Dou et al., 2003; O'Donnell et al., 2006) and as a histopathological correlate of HIV-induced behavioral abnormalities (Cook-Easterwood et al., 2007; Sas et al., 2007). Results from analysis of MAP2-stained frontal cortex regions parallel the behavioral findings. Loss of MAP2 staining produced by clade C HIV-1 is intermediate compared with clade B and control mice. Astroglia, a key pathological feature of HAD, was significantly greater ($p = 0.0117$) in clade B HIVE mice than in clade C HIVE mice. Similar numbers of p24-positive cells in the brains and equivalent copy numbers of viral RNA in brain sections of both types of mice confirmed equal viral loads in both cases. These findings not only suggest that clade differences play a major role in pathogenesis of HAD, but also highlight the usefulness of the SCID HIVE mouse model in detecting subtle differences in behavior and histopathology.

More recently it has been shown that direct neurotoxicity of Tat may be mediated by Tat's binding to NMDA receptor subunits NR1 and 2A (A. Nath, personal communication) and that clade C or a C31S mutant of clade B Tat protein does not cause neurotoxicity (Li et al., 2005), providing an additional explanation for clade differences in NeuroAIDS. The mechanism of toxicity was not mediated by a complex formation among lipoprotein receptor-related protein (LRP), PSD-95, NMDAR (NMDA receptor), and nNOS (neuronal nitric oxide synthase) on the surface of human neurons, as we described previously (Eugenin et al., 2007). It was also not attributable to LRP-mediated Tat internalization, as described in neurons (Liu et al., 2000), because RAP (receptor-associated protein), an LRP blocker, did not abrogate toxicity. These differences in Tat-mediated toxicity may be attributable to the low expression of LRP receptor on HEK cells and the absence of PSD-95 to bridge NMDA receptors and LRP.

Regardless of the precise mechanism of Tat-mediated effects on neurons, our results show that behavioral and neuropathological differences between HIV-1 clades are attributable to an attenuated ability of clade C Tat to cause neurotoxicity by both direct and indirect mechanisms. Although the studies by A. Nath and colleagues (W. Li, Y. Huang, R. Reid, T. Malpica, J. Steiner, S. Shankar, A. Mahadevan, P. Satishchandra, A. Nath, personal communication) point to clade differences in NMDA receptor-

mediated apoptosis, Campbell et al. (2007) showed that clade C Tat protein is defective in its ability to generate an intracellular calcium influx and in inducing TNF- α , a cytokine that promotes astrogliosis. Additionally, Mishra et al. (2008) has reported lower neurotoxicity, a significantly reduced ROS (reactive oxygen species) induction, and weaker mitochondrial membrane polarization in human neurons by clade C Tat.

These neurotoxic effects of clade B HIV-1 may have implications for both neuronal and astrocytic dysfunction in HIVE in SCID mice and HAD in humans. The increased astrogliosis seen in HIVE mice with clade B HIV-1 compared with C reflects a larger pool of dysfunctional astrocytes observed (Lipton and Gendelman, 1995). Normally, astrocytes are responsible for clearing excess glutamate at the synaptic cleft to prevent NMDA receptor-mediated excitotoxicity. However, if astrocytes are impaired in this function, it could explain why neurons in the area of greatest astrogliosis display decreased dendritic arborization. In addition, neurotoxicity in clade B mice could have more direct effects on neurons that are in the vicinity of HIV-infected human MDMs, adding to (indirect) astrocyte effects on neuronal dendritic formation. Therefore, because frontal cortical dendritic formation is important to learned behavior, the clade B mice make a greater number of errors in the WRAM.

Previously, we showed that clade C Tat is defective in monocyte chemotaxis using purified Tat protein *in vitro* (Ranga et al., 2004). Here, we used HIV-1-infected MDMs for the recruitment of monocytes to demonstrate that monocyte chemotaxis occurs in the setting of virus-infected cells. In comparing the two clades of HIV-1, the differences observed were not caused by differential virus growth. Under these conditions, clade C HIV-infected cells were able to recruit significantly reduced numbers of monocytes compared with clade B HIV-infected MDMs. The SCID HIVE model used here does not allow us to examine whether the differential monocyte chemotaxis observed *in vitro* applies to monocyte migration across the blood–brain barrier *in vivo*. Therefore we are looking into developing modified mouse models, in which one can observe the migration of macrophages introduced in the periphery to the sites in brain where HIV-infected macrophages reside under conditions that transiently break down the blood–brain barrier.

In verifying that the monocyte chemotaxis induced by HIV-infected MDMs is truly mediated by Tat protein, we used anti-Tat antibodies with a proven ability to neutralize both clade B and clade C Tat proteins (Siddappa et al., 2006). Interestingly, neutralizing the Tat protein present in the spent media lowered monocyte migration to control levels, and neutralizing the CCL2 was also effective in blocking monocyte migration, suggesting that Tat or HIV infection induces the secretion of CCL2, which then mediates monocyte migration. These novel findings demonstrate a direct role for Tat during HIV infection of MDMs in monocyte migration. CCL2 is presumably produced as a result of HIV-1 infection. Media from clade C HIV-infected MDMs also showed reduction in the migrating monocytes with anti-Tat and anti-CCL2 antibodies. This reduction was lower than that observed with clade B, but statistically significant ($p = 0.008$ for anti-Tat and $p = 0.005$ for anti-CCL2).

The ability of clade B HIV-1 Tat protein as well as clade B HIV-1 virus to induce MDMs and astrocytes to secrete CCL2 points to an indirect means by which HIV infection leads to enhanced monocyte infiltration into the brain. We took advantage of the availability of Tat preparations that were equally biologically active in their ability to be taken up by cells and to activate HIV-1 LTR-mediated expression of a GFP reporter (Sid-

dappa et al., 2006). Despite their similar activities (Siddappa et al., 2006), there were significant differences in CCL2 induction by the two different Tat proteins when incubated with primary astrocytes or MDMs. These differences were also observed between MDMs infected with the two clades of HIV-1. The differential induction of CCL2 by the two Tat proteins and by infection with the two clades of HIV-1 suggests another key difference between the clades in the pathogenesis of HAD.

Our results demonstrate that genotypic differences between HIV-1 clade B and clade C viruses do translate to phenotype differences in HAD pathogenesis outcomes *in vivo* by virtue of decreased direct neurotoxicity of clade C HIV-1 as well as lead to differences observable *in vitro*, including defective monocyte migration and the impaired secretion of chemotactic proteins such as CCL2. Our results provides a concrete proof of interclade differences and hope that specific HIV genes directly involved in neuropathogenesis can be pinpointed by the use of chimeric viruses between clades, work on which is currently being pursued in our laboratory. The findings reported here may have significant implications for future treatment strategies in HAD by helping target specific genes responsible for pathogenesis of HAD.

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