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Development of Neurological Disease Is Associated with Increased Immune Activation in Simian Immunodeficiency Virus-Infected Macaques

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Simian immunodeficiency virus (SIV) infection of macaques can result in central nervous system disorders, such as meningitis and encephalitis. We studied 10 animals inoculated with brain-derived virus from animals with SIV encephalitis. Over half of the macaques developed SIV-induced neurologic disease. Elevated levels of systemic immune activation were observed to correlate with viral RNA in the cerebral spinal fluid but not with plasma viral load, consistent with a role for SIV in the pathogenesis of neurologic disease.

Neurotropism and neurovirulence by human immunodeficiency virus type 1 (HIV-1) are still poorly understood despite an intense research focus. Early after systemic infection, evidence of HIV-1 invasion into the central nervous system (CNS) has been shown (6). Although HIV-1 is present in the brain during primary infection, ~25% of HIV-infected patients will develop neurological disease years later, during AIDS (13). The basis for the delayed and incomplete penetrance of CNS manifestations of HIV disease, despite early virus invasion of the CNS, remains unclear. Interestingly, this same phenomenon is recapitulated in key aspects in macaques infected with simian immunodeficiency virus (SIV) (9), although the disease course in macaques is often more rapid.

We recently described two macaques that developed SIV-induced encephalitis (SIVE) later in the disease course (conventional progressors) (4). Phylogenetic analyses of envelope sequences from viruses isolated from the cerebral spinal fluid (CSF) and plasma from these animals demonstrated tissue compartmentalization, consistent with adaptive evolution of the virus in the central nervous system (CNS) (4). Virus from one of these macaques (H631) was isolated from cryopreserved brain samples collected at necropsy following saline perfusion to minimize blood contamination.

(This work was previously presented in part at the 2010 Nonhuman Primate Models for AIDS conference in New Orleans, LA.)

To evaluate the role of viral neurotropism and neurovirulence in the development of SIV-associated neurological disease, we intravenously inoculated 500 50% tissue culture infective doses ($TCID_{50}$) of H631 brain-derived virus into four rhesus macaques (H761, H780, H782, and H783). During acute infection, peak viral loads ranged from 10^4 to 10^6 and 10^2 to 10^5 copies/ml in plasma and CSF, respectively (Table 1 and Fig. 1A and B). Animals H761 and H780, which expressed Mamu-A*01, a major histocompatibility complex class I (MHC-I) allele associated with more effective control of viremia, were eventually able to control virus replication and were euthanized at the conclusion of the experiment. These animals had no evidence of SIV-related disease, including SIVE. However, the two other animals, H782 and H783, remained viremic and subsequently succumbed to AIDS with similar terminal plasma viral loads (Fig. 1A). Interestingly, despite both animals having modest to high peak CSF viral loads during acute infection, only H783 maintained robust viral replication in the CNS, as measured by the terminal CSF (Table 1 and Fig. 1B).

Histopathologic examination of the brain from animal H783 revealed severe, multifocal SIVE that was characterized by perivascular accumulation of macrophages, lymphocytes, and multinucleated giant cells (MNGC), similar to lesions described earlier in animal H631 (4). *In situ* hybridization (ISH) for SIV and confocal microscopy identified SIV-expressing cells as macrophages in the meninges and brain, the latter representing the pathological hallmark of SIVE (Fig. 1C). To further adapt this virus, the virus was isolated from the brain of H783, and 500 $TCID_{50}$ of virus was then inoculated intravenously into six naïve rhesus macaques that did not express any of the known restrictive MHC-I alleles.

All macaques inoculated with this passaged virus became infected with peak and setpoint plasma viral loads ranging from 10^5 to 10^7 and 10^4 to 10^6, respectively (Fig. 1A). Peak virus loads in the CSF ranged from 10^3 to 10^5 copies/ml, and although levels declined initially after primary infection, increasing levels were observed in 5 of the 6 animals, sometimes to levels greater than observed concurrently in plasma (Table 1 and Fig. 1A and B). H803 was sacrificed at 6 months due to non-SIV-related problems prior to development of AIDS, but the animal showed mild, focal lesions in the meninges and parenchyma of the brain. Among the remaining five macaques, the speed of disease progression was variable (13 to 101 weeks), but all animals eventually developed

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TABLE 1 Summary of CNS disease outcomes and SIV RNA viral loads[a]

Animal no.	Inoculum (brain-derived virus from:)		Meningitis	Encephalitis	CSF viral load (log_{10}) at:		Plasma viral load (log_{10}) at:		Survival (wks)
	H631	H783			Peak	Death	Peak	Death	
H761	X				3	2	5	2	78
H780	X				2	2	4	3	72
H782	X				4	2	6	5	70
H783	X		X	X	5	7	6	5	43
DBTG		X			4	3	6	5	101
DBTN		X	X	X	5	6	7	4	13
H801		X	X		3	7	6	5	54
H802		X	X	X	3	6	6	6	18
H803		X	X	X	5	5	5	6	24[b]
H804		X	X	X	3	7	6	7	43

[a] Other SIV-related lesions included lymphoma (animal H782), enteritis (H782, DBTG, DBTN, and H804), *Pneumocystis* pneumonia (DBTN and H801), and *Mycobacterium avium* infection (H801).

[b] Animal H803 was euthanized early due to non-SIV-related issues; there were no opportunistic infections but there was the presence of mild meningoencephalitis.

AIDS-related illnesses (Table 1) and were euthanized. Three of these animals (DBTN, H802, and H804) also developed terminal neurologic signs that included head tilt, ataxia, motor difficulties, and tremor. Examination of brain tissues obtained at necropsy by histopathology, SIV-specific ISH, and confocal microscopy demonstrated SIV-infected MNGC of macrophage origin in the meninges in four of these five animals; the three macaques with neurologic signs were also determined to have SIVE (Table 1), as shown by perivascular accumulations of SIV^+ cells within the brain parenchyma (Fig. 1D). Thus, considering animals inocu-

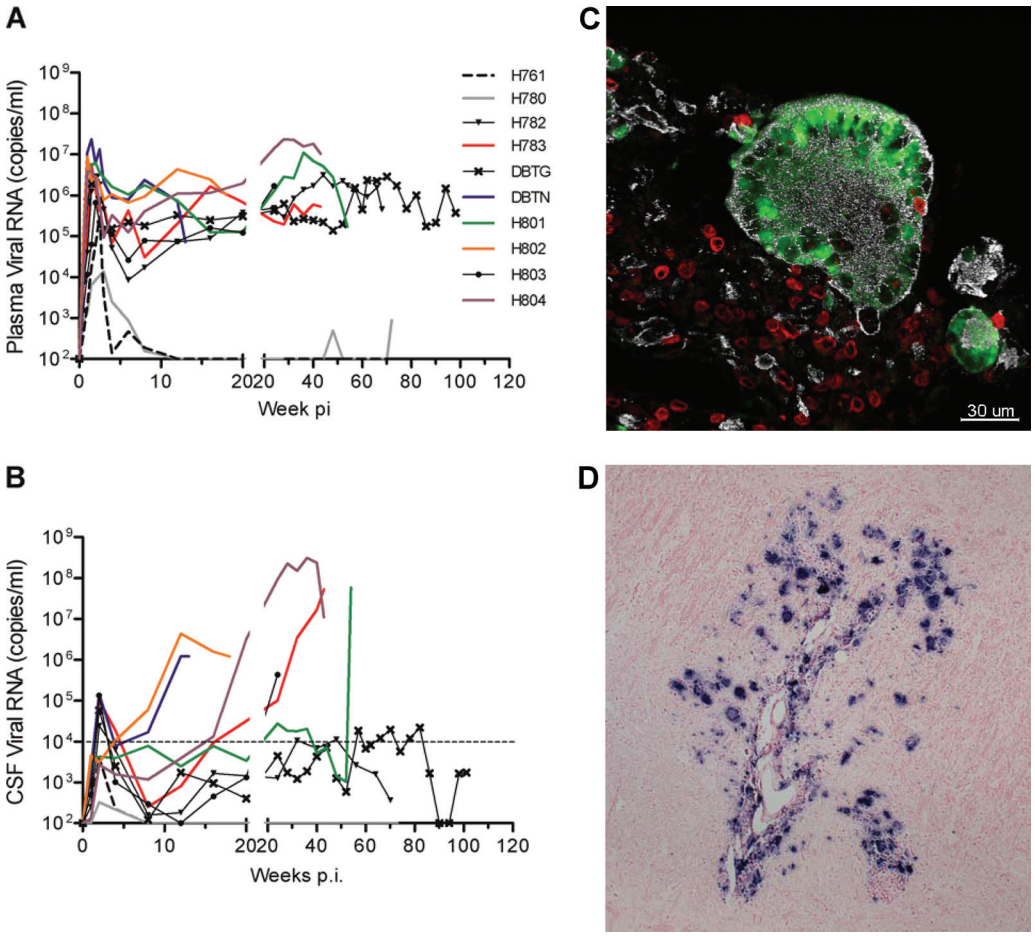

FIG 1 Viral RNA levels in plasma (A) and CSF (B). Animals H761, H780, H782, and H783 were inoculated with H631-derived brain virus. Animals DBTG, DBTN, and H801 to H804 were inoculated with H783-derived brain virus. Cell-free virus stock was generated by incubating cryopreserved brain tissue with rhesus peripheral blood mononuclear cells. Culture supernatant was then filtered, and the $TCID_{50}$ was determined in a TZMbl cell assay. p.i., time postinfection. (C) Confocal microscopy of a brain section from H783 cells stained for CD3 (red), Ham56 (white), and SIV ISH (green), demonstrating the presence of SIV RNA in macrophages. (D) SIV-specific *in situ* hybridization, showing a typical perivascular lesion in the brain of macaque H804. ISH and confocal microscopy were performed as described previously (4). All animals were specific pathogen free (i.e., free of simian T cell leukemia virus, SIV, and simian retrovirus).

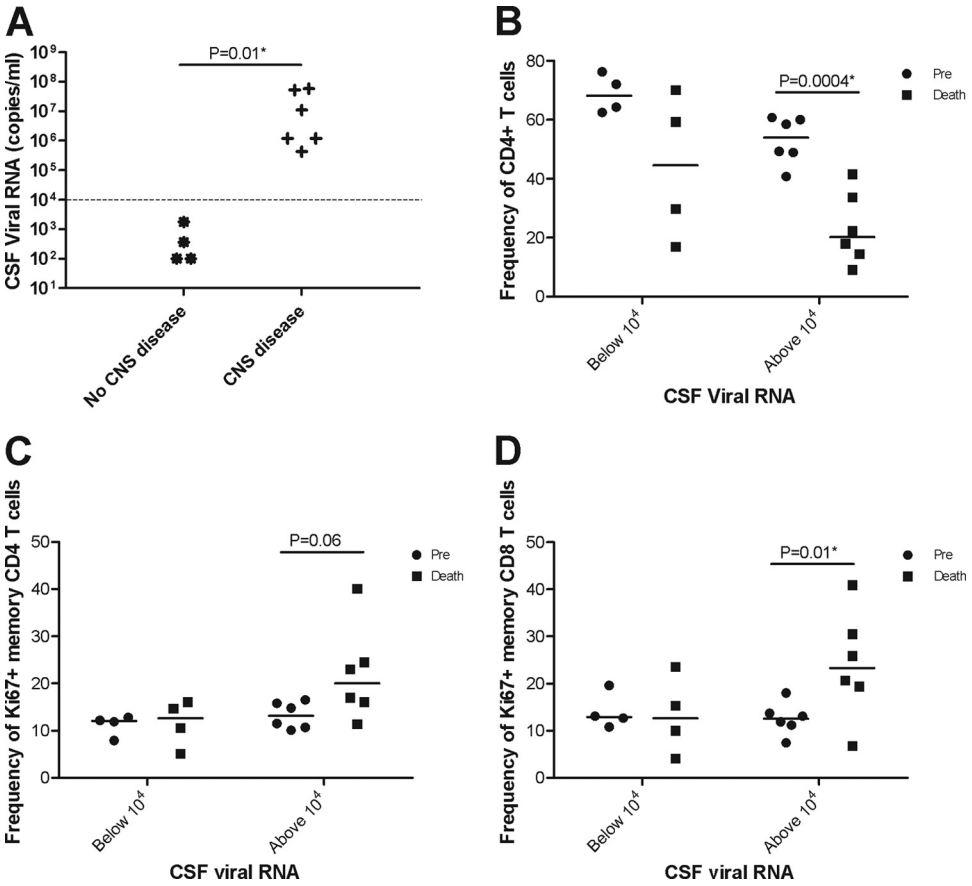

FIG 2 Disparity in CSF viral RNA levels associated with neurological disease. (A) Terminal CSF viral loads of greater than 10^4 copies/ml were associated with evidence of CNS disease ($P = 0.01$). (B) Animals with high CSF viral RNA levels had significantly lower CD4 T cells by the time of death ($P = 0.0004$). (C and D) The frequencies of Ki67$^+$ memory CD4 (C) and CD8 (D) T cells were higher in animals with high terminal CSF viral loads. Circles represent samples prior to inoculation, and squares represent samples at death. Horizontal bars represent median values. The Mann-Whitney U *t* test (A) and a paired *t* test (B to D) (Prism v. 5.04, GraphPad Software, Inc.) were performed to determine statistical significance. Peripheral blood mononuclear cells were stained with the following reagents: CD3 (557917; BDPharmingen), CD8 (558207; BDPharmingen), CD4 (35004873; eBioscience), Ki67 (556026; BDPharmigen), CD95 (559773; BDPharmingen), CD14 (IM2580U; Beckman Coulter), CD28 (6607111; Beckman Coulter), CD20 (25020973; eBioscience), CCR5 (550632; BDPharmingen), and Aqua Live/Dead (L34957; Invitrogen). CD4 or CD8 T cells were defined as CD3$^+$/CD4$^+$ or CD3$^+$/CD8$^+$. Ki67$^+$ memory CD4 T cells were defined as CD3$^+$/CD4$^+$/CD95$^+$/Ki67$^+$. Similarly, Ki67$^+$ memory CD8 T cells were defined as CD3$^+$/CD8$^+$/CD95$^+$/Ki67$^+$. Multicolor flow cytometry was performed using a BD-LSRFortessa cell analyzer with the DiVa software (v. 6.0), collecting ~10^6 events per sample. FlowJo software (v. 9.3; TreeStar) was used for data analysis.

lated with either of these two viruses (SIVsmH631Br or SIVsmH783Br), 6 of 10 macaques inoculated with brain-derived virus isolated from animals with SIVE developed neurological disease (Fig. 1B and 2A). This incidence of neurologic disease was significantly increased from that reported in prior studies with the original parental SIVsmE543-3, in which SIVE was only observed rarely (data not shown). In agreement with HIV and SIV studies that have found a relationship between high CSF viral load and CNS disease, histologic evidence of neuropathology and SIV replication in the CNS was seen only in animals with a minimum of 10^4 copies/ml in the CSF at the time of necropsy ($P = 0.01$) (Fig. 2A).

It has been well established that in HIV/SIV infection, neurological disorders appear late in disease, in association with immunosuppression. Consistent with this, animals in our study with CSF viral loads above 10^4 and CNS disease had significantly lower numbers of CD4$^+$ T cells at death ($P = 0.0004$) than those that had low CSF viral loads and did not have neurologic disease (Fig. 2B). A strong predictor of disease progression is chronic immune activation (2), which has been found to be independent of CD4 T cell counts (10, 11). Immune activation can be directly detected by measuring markers of cellular activation, such as HLA-DR, CD38, and CD69. Alternatively, the expression of Ki67, a marker for cellular proliferation, can also be used as a reflection of immune activation. Indeed, Ki67 levels have been found to correlate with expression HLA-DR, CD38, and CD69 in HIV$^+$ individuals (12), and furthermore, a significant correlation was observed for Ki67$^+$ CD4 or CD8 T cells with rate of disease progression (5).

Therefore, in this study, we used Ki67 as a surrogate marker for activation, since activation is required for proliferation. In animals that had terminal CSF viral loads of less than 10^4 copies/ml, there were no differences in the frequencies of proliferating CD4 or CD8 memory T cells, measured preinfection and at death (Fig. 2C and D). However, in animals whose CSF viral loads were greater than 10^4 copies/ml at the time of euthanasia, the frequencies of proliferating CD4 and CD8 memory T cells increased between the preinfection time point and euthanasia. This difference

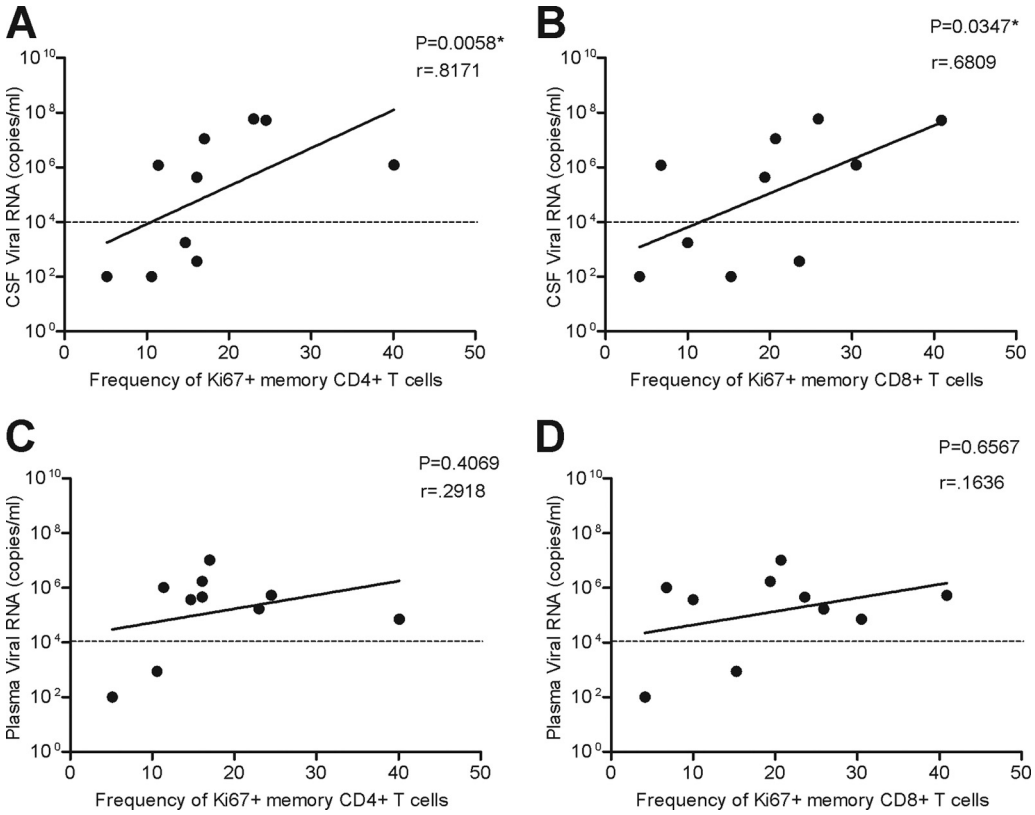

FIG 3 Correlations of CSF viral load and immune activation with neurological disease. (A and B) The higher frequencies of Ki67$^+$ memory CD4 T cells (A) and CD8 T cells (B) significantly correlated with a higher CSF viral load ($P = 0.0058$ and 0.0347, respectively). Interestingly, the plasma viral loads did not correlate with the frequencies of Ki67$^+$ memory CD4 T cells (C) or CD8 T cells (D). *P* and *r* values were calculated using Spearman rank correlation analyses, and diagonal lines represent the linear regressions. All statistical analyses were performed with Prism v. 5.04 (GraphPad Software, Inc.).

approached statistical significance for Ki67$^+$ CD4 memory T cells ($P = 0.06$) and was statistically significant for Ki67$^+$ CD8 memory T cells ($P = 0.01$) (Fig. 2C and D). Indeed, the immune activation status in blood was significantly correlated with CSF viral load (Spearman $r = 0.8171$, $P = 0.0058$ [Fig. 3A]; Spearman $r = 0.6809$, $P = 0.0347$ [Fig. 3B]). Interestingly, we observed no correlation between systemic immune activation and plasma viral load at necropsy (Fig. 3C and D).

Unlike other models which have used pig-tailed macaques as a rapid, high-incidence model of SIV-induced neurological disease, we chose to use rhesus macaques to more accurately represent the more variable disease course observed in humans. Uninfected pig-tailed macaques have been shown to have higher levels of memory effector CD4 and CD8 T cells and immune activation than uninfected rhesus macaques (8). Thus, the increase in the baseline frequency of initial available target cells and immune activation status may contribute to the greater likelihood and severity of CNS disease in these models. Moreover, it has been suggested that pig-tailed macaques are more susceptible to SIV-induced CNS disease than rhesus macaques (14). In contrast, a conventional disease progression model in the latter species can better recapitulate what occurs in HIV-infected humans and, additionally, affords the ability to study both virus as well as host factors important to neurological disease.

Using such a model, we found that immune activation positively correlated with viral load in the CSF but not with plasma viral load. It has been shown that microbial translocation leads to pathological, chronic, systemic immune activation (3). Interestingly, a link between immune activation and plasma lipopolysaccharide levels in patients with HIV-associated dementia was recently demonstrated (1), although associations between viral load in CSF and immune activation were not evaluated. In the present study, we measured the plasma level of lipopolysaccharide binding protein at death, but we did not detect a significant difference between animals with high versus low CSF viral loads (data not shown). However, it should be noted that in the previously published study (1), a large number of patients with and without neurocognitive impairment were evaluated to detect this correlation. A discrepancy between these studies may reflect the small number of animals evaluated in the present study.

The correlation between immune activation and viral load in the CSF in our study does not indicate whether immune activation is a cause or consequence of SIVE, i.e., whether systemic immune activation drives the development of SIVE or whether the local inflammatory process in the CNS promotes systemic immune activation. In favor of the latter interpretation, virus replication in the brain appeared to outpace that outside the CNS, as observed with significantly higher viral loads (1- to 2-log steps) in the CSF versus plasma at the time of death of animals with SIVE (Table 1). Additionally, this association is interesting in light of the persistence of immune activation in people on antiretroviral therapy (ART) in whom plasma viremia has been effectively suppressed

(7). Many of the drugs used in ART do not efficiently cross the blood-brain barrier. Potential reservoirs of virus replication in the CNS may contribute to the persistent immune activation in these patients. Studies of CSF viral load in such patients would therefore be of considerable interest.

Nevertheless, other factors must be involved, as not all patients nor rhesus macaques with AIDS develop neurological disease, even though there is initial viral replication in the CNS. Neurovirulence of the virus is likely an important feature, but in preliminary sequence analyses, we were unable to identify a brain-specific signature common to all animals (data not shown). This was not unexpected, as there is considerable divergence in *env* sequences between the CNS and periphery (4) as well as between individual animals, particularly in those inoculated with different virus lineages. Clearly, the extent of replication in the CNS is an important predictor, since evidence of CNS pathology was not seen in animals with CSF viral RNA levels less than 10^4 copies/ml.

In summary, although further work will be necessary to dissect the virus-specific factors leading to HIV/SIV-associated CNS disease, the results shown here demonstrate the complex interplay between host response, such as immune activation, and virus infection that is important to development of neurological disease.

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