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Brain CD8+ and cytotoxic T lymphocytes are associated with, and may be specific for, human immunodeficiency virus type 1 encephalitis in patients with acquired immunodeficiency syndrome

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CD8+ T cells infiltrate brains with human immunodeficiency virus type-1 (HIV-1) encephalitis (HIVE) and related animal models; their perineuronal localization suggests cytotoxic T cell (CTL)-mediated neuronal killing. Because CTLs have not been identified in acquired immunodeficiency syndrome (AIDS) brains, the authors identified their cytotoxic granules in autopsy AIDS brains with HIVE and without HIVE (HIVnE) plus controls (7 to 13 cases/group) and determined gene expression profiles of CTL-associated genes in a separate series of cases. CD3+ and CD8+ T cells were significantly increased ($P < .01$) in perivascular spaces and inflammatory nodules in HIVE but were rare or absent in brain parenchyma in HIVnE and control brains. Eight HIVE brains contained granzyme B+ T cells and five contained perforin+ T cells. Their T-cell origin was confirmed by colocalization of CD8 and granzyme B in the same cell and the absence of CD56+ natural killer cells. The CTLs directly contacted with neurons, as the authors showed previously for CD3+ and CD8+ T cells. CTLs were rare or absent in HIV nonencephalitis (HIVnE) and controls. Granzyme B and H precursor gene expression was up-regulated and interleukin (IL)-12A precursor, a maturation factor for natural killer cells and CTLs, was down-regulated in HIVE versus HIVnE brain. This study demonstrates, for the first time, CTLs in HIVE and shows that parenchymal T cells and CTLs are sensitive biomarkers for HIVE. Consequently, CD8+ T cells and CTLs could mediate brain injury in HIVE and may represent an important biomarker for productive brain infection by HIV-1. *Journal of NeuroVirology* (2006) **12,** 272–283.

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Introduction

Children and adults with human immunodeficiency virus type-1 (HIV-1) infection are at risk to develop a progressive encephalopathy or dementia (HIV-1–associated dementia [HAD]) in the setting of immunosuppression and acquired immunodeficiency syndrome (AIDS). Rarely, HAD is the AIDS-defining illness. Prior to the introduction of antiretroviral therapy in the late 1980s, the incidence of HAD ranged from 20% to 50%. The use of combined highly active antiretroviral therapy (HAART) 10 years later delayed the onset of neuropsychological impairment and slowed the progression of symptoms, changing HAD from a subacute to a more chronic illness (McArthur *et al*, 2003; Brew *et al* 2004). Nevertheless, the beneficial effects of HAART on neurocognition are lost once systemic viral loads no longer can be suppressed (Dore *et al* 1999; McArthur *et al*, 2002).

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The pathological substrate of HAD is HIV-1 encephalitis (HIVE) (Cherner *et al*, 2002), an inflammatory brain infection where on-going viral production is confined to cells of monocyte origin, such as perivascular macrophages, microglia, and the pathonomonic multinucleated giant cell of HIVE. The brain infection concentrates in subcortical regions, especially basal ganglia and hippocampus, temporal lobe white matter, and centrum semi-ovale. Neuronal and synaptic loss and focal or diffuse white matter atrophy accompany, and are likely due to, HIVE. Despite the effectiveness of HAART in reducing or eradicating systemic viral load and delaying the onset of HAD, many large autopsy series of end-stage AIDS patients suggest that the incidence of HIVE at the time of death is unchanged from that encountered in the pre-retroviral therapy era (Jellingery *et al*, 2000; Neuenburg *et al*, 2002; Gray *et al*, 2003).

The mechanisms of brain injury in HIV-1 infection may be multiple. Macrophage-induced neurotoxicity is considered by many to be paramount due to the predominance of activated microglia and monocytes; their known secretion of a variety of neurotoxins; and the *in vivo* brain restriction of productive infection to this cell type (Epstein and Gendelman, 1993). Certain HIV-1 proteins, specifically gp120 and transcriptional activator protein (Tat), also may contribute to brain damage because both are neurotoxic *in vitro* and following intraparenchymal or intraventricular brain injection (Singh *et al*, 2004). Restricted HIV-1 infection of reactive astrocytes adds another potential mechanism for brain dysfunction and injury because these cells play a vital role in maintaining the normal function of neurons and of the blood-brain barrier (Brack-Werner, 1999). Latent neuronal infection, with its attendant disruption of normal cell functioning, is suggested by two of five studies that amplified HIV-1 sequences in these cells by *in situ* polymerase chain reaction (PCR) (Nuovo *et al*, 1994; Bagasra *et al*, 1996) and by two later studies that detected amplified viral sequences in DNA extracted from neuronal nuclei removed from tissue sections by laser-assisted microdissection (Torres-Muñoz *et al*, 2001; Trillo-Pazos *et al*, 2003).

T lymphocytes, which comprise part of the inflammatory response in HIVE and HAD (Navia *et al*, 1986), may represent an additional mechanism of brain injury in AIDS brains. CD8+ T cells infiltrate perivascular spaces, inflammatory nodules, and brain parenchyma in AIDS patients with HIVE (Petito *et al*, 2003) and accompany the inflammatory response in simian immunodeficiency virus (SIV) infection and encephalitis (Marcondes *et al*, 2001; Kim *et al*, 2004). Previously, we showed that significant increases in parenchymal CD3+ T cells were confined to brain regions of local HIVE (Petito *et al*, 2003), a finding that suggests that their recruitment or retention in brain is specifically related to productive viral infection. Additionally, we found that CD4+ cells were more common in the perivascular infiltrates than brain parenchyma whereas CD8+ T cells were present in both locations.

Several lines of investigation suggest that T cells damage brain in HIV-1 and SIV-infections. First, SIV-infected macaques have elevated gene expression levels for the cytotoxic lymphocyte (CTL) cytotoxic granules, granzymes A and B, perforin, and interferon (IFN)-γ (Marcondes *et al*, 2001). Second, two AIDS patients either developed, or experienced, a worsening of HAD subsequent to HAART-induced immune reconstitution; at autopsy, large numbers of CD8+ T cells infiltrated brain tissue (Miller *et al*, 2004). Lastly, infiltrating CD8+, as well as CD3+, T cells form direct cell-to-cell contact with neurons in brains of patients with HIVE (Petito *et al*, 2003), thereby providing the anatomical relationship required for CTL-mediated cell killing. Because CTL-mediated cell killing requires direct contact with target cell, this observed T-cell contact with neurons is highly consistent with CTL-mediated cell death in HIVE.

In this present study, we determined the presence and anatomical relationship between CD8+ T cells and CTLs in postmortem brains of AIDS patients with and without HIVE, plus uninfected controls matched for age and postmortem intervals. We paid specific attention to their distribution in perineuronal spaces and cerebral white matter. We used commercially available monoclonal antibodies directed against perforin and granzyme B as markers of activated CTLs; CD3 and CD8 to label these T-cell subsets; and CD56 (neural cell adhesion molecule) to label natural killer (NK) cells because NK cells also are cytotoxic and express granzyme B and perforin. We examined the differential gene expression of CTL-associated genes in fresh-frozen temporal lobe cortex obtained at autopsy from a separate group of HIVE and HIV nonencephalitis (HIVnE) AIDS brains. A portion of this work has been presented in abstract form (Torres-Muñoz *et al*, 2004a).

Results

The mean age and postmortem interval (PMI) of the patient groups was similar within each study ($P > .05$) (Table 1). The lower PMI of the gene expression study reflects our accrual policy that limits collection of frozen samples to cases with PMIs of less than 24 h. One of the immunohistochemical (IHC) AIDS cases initially classified as HIVnE was placed into the HIVE group when its moderate CD3+ lymphocytosis prompted review of its autopsy slides; at that time, a solitary multinucleated cell characteristic of HIVE was detected.

The AIDS groups contained more men and fewer Caucasians (not shown) than controls, consistent with the gender and racial difference in our local HIV-1–infected population. Brain pathology other than HIVE included subdural hematoma, remote toxoplasmosis, acute hypoxic-ischemic encephalopathy,

Table 1 Patient demographics in AIDS patients with and without HIVE and normal controls

Group	*Study (and group no.)*	*Age (years)*	*Gender*	*PMI (hrs)*	*Non-HIVE neuropathology*
HIVE	IHC (9)	43.1 ± 10.9	7 men; 2 women	18.1 ± 10.1	Subdural hematoma (1)
HIVnE	IHC (7)	48.4 ± 9.7	7 men	20.4 ± 4.6	HIE, acute
Control	IHC (13)	42.8 ± 8.4	6 men; 7 women	24 ± 11.8	Lacunes, remote (2)
HIVE	Gene expression (5)	37.0 ± 7.7	3 men; 2 women	5.9 ± 2.4	Toxoplasmosis, remote (2)
HIVnE	Gene expression (4)	37.8 ± 10.1	3 men; 1 woman	11 ± 5.7	Carcinomatous meningitis (1)

Abbreviations: HIVE: AIDS with HIV-1 encephalitis; HIVnE: AIDS without HIVE; IHC: immunohistochemical study; HIE: hypoxic-ischemic encephalopathy, focal. Numbers in parenthesis refer to number of cases. Data is are expressed as mean ± SD and significant differences determined with ANOVA and Bonferroni multiple comparison test. $P > .05$ for age and PMI intergroup differences.

carcinomatous meningitis, and brain lacunes. Except for the acute hypoxic-ischemic changes, all pathologies were in brain regions remote from those selected for these studies. Most of the HIVE and HIVnE cases were end-stage AIDS cases with systemic opportunistic infections or lymphomas. Clinical information regarding HAART, peripheral lymphocyte counts, and neurological status was limited in most cases to the terminal hospitalization records which usually did not detail these parameters.

Perivascular T cells

CD3+ perivascular T cells were significantly increased in hippocampus and basal ganglia of HIVE brains compared with HIVnE and controls ($P < .01$), whereas the numbers of perivascular CD3+ cells were similar in HIVnE and controls ($P > .05$) (Table 2, Figure 1A). CD8+ T cells were more numerous in both HIVE (Figure 1A) and HIVnE compared with controls, with the CD8 grade averaging 1.56 ± 0.24 and 1.43 ± 0.20 in HIVE and HIVnE, respectively, versus 1.0 ± 0 for controls. Differences were not significant with Kruskal-Wallis test, although HIVE and HIVnE perivascular CD8+ T cells were significantly higher than controls ($P < .01$) but not from each other ($P > .05$) with ANOVA and the Bonferoni test. The numbers of perivascular CD8+ T cells were lower than perivascular CD3+ T cells in the HIVE cases (Figure 1A), a finding consistent with our prior study that showed that perivascular T cells contained both CD4+ and CD8+ T cells whereas intraparenchymal T cells were primarily CD8+ (Petito *et al*, 2003).

Parenchymal T cells

Focal and diffuse collections of CD3+ and CD8+ T cells (Figure 2) and CD8+ T cells were prominent in HIVE brains in the CA4 region of the hippocampus (Figure 2A) and temporal lobe white matter (Figure 2B) but less frequent in temporal lobe cortex. With the exception of one HIVnE case (see below), they were rare or absent in HIVnE and controls. When we used a grading scale to measure their numbers (Table 2), the differences were highly significant for CD3+ T cells ($P < .01$) with the Kruskal-Wallis test for nonparametric data but only significantly different for the CD8+ T cells with ANOVA, a likely consequence of the zero value for the control CD8+ T cells. When we counted the number of CD8+ T cells per 100× microscopic field, we found that the CA4 region of the hippocampus contained the highest concentration of parenchymal CD8+ cells, followed by the temporal lobe white matter and then the cerebral cortex (Figure 1B). CD8+ T cells were located in perivascular spaces (Figure 3A) and inflammatory nodules (Figures 4 and 5A). As previously reported (Petito *et al*, 2002), CD8+ T cells also formed direct cell-to-cell contacts with neuronal cell bodies (Figure 5A) in six of nine HIVE cases.

One of seven HIVnE brains contained a moderate, grade 2, number of parenchymal CD3+ and CD8+ cells, with the actual number of CD8+ T cells/unit area measuring 36 in subcortical white matter, 3 in the CA4 region of the hippocampus, and 0.9 in temporal lobe cortex. Although we were unable to find any brain infection in the available microscopic

Table 2 Distribution of CD3+ and CD8+ T cells and cytotoxic cells in brains of AIDS and control patients

	Perivascular T lymphocytes		*Parenchymal T cells*		*Number of cases with CTL granule-positive lymphocytes*	
	CD3+	*CD8+*	*CD3+*	*CD8+*	*Perforin+*	*Granzyme B+*
HIVE	2.12 ± 0.17*	1.56 ± 0.24	2.12 ± 0.17**	2.22 ± 0.31[a]	5 of 9	8 of 9
HIVnE	1.07 ± 0.13	1.43 ± 0.2	0.4 ± 0.0.1	0.57 ± 0.3	0 of 1	1 of 7[a]
Control	0.85 ± 0.1	1 ± 0	0.38 ± 0.13	0 ± 0	n.d.	0 of 5

Note. CD3+ and CD8+ T cells are measured with a sliding scale from 0 to 3+ and significance determined with the Kruskal-Wallis test (*) or ANOVA ([a]). One HIVnE patient had 2+ perivascular inflammation and rare granzyme B+ cells in the white matter (see Figure 1).
*$P < .01$ compared with HIVnE and $P < .001$ compared with controls; **$P < .001$ compared with HIVnE and $< .01$ compared with controls.
[a]$P < .001$ compared with HIVnE and controls with ANOVA.

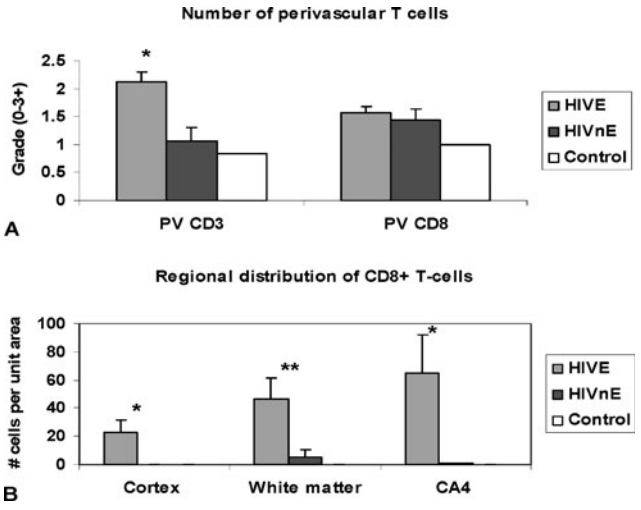

Figure 1 **A** Perivascular CD3+ T cells are more numerous in HIVE than in HIVnE and controls ($P < .01$), whereas the numbers of CD8+ T cells are only slightly increased ($P > .05$). *Y* axis: CD3+ and CD8+ T lymphocyte infiltrates measured from 0 to 3+ and expressed as mean ± SE. $^{*}P < .01$ compared to HIVnE and < .001 compared to controls, as determined with the Kruskal-Wallis test. **B**, The regional distribution of CD8+ T cells in HIVE brains per unit area (100× microscopic field) is highest in the CA4 region of the hippocampus, intermediate in temporal lobe white matter, and lowest in temporal lobe cortex. All three regions contain significantly more CD8+ T cells than either HIVnE or controls. *Y* axis: Number of CD8+ T cells per 100× microscopic field expressed as mean ± SE. $^{*}P < .05$ compared to HIVnE and controls; $^{**}P < .05$ compared to HIVnE and < .01 compared to controls; as determined with ANOVA and Bonferroni multiple comparison test.

slides of this case, the presence of the CD3+, CD8+, and granzyme B+ cells suggests that HIVE may have been present in tissues not sampled for histology.

Granzyme B- or perforin-positive cells readily were identified in perivascular spaces (Figure 3B), inflammatory nodules (Figures 4 and 5B) and perineuronal spaces (Figures 5B and 6) in those regions with moderate numbers of CD8+ T cells. Their numbers were proportional to, although slightly less than, the numbers of CD8+ cells in the same region. The small size of the granzyme B+ and perforin+ cytoplasmic granules compared with the cytoplasmic membrane staining of the CD8 antigen reduced the apparent number of the CTLs. When we counted the numbers of each cell type in a given region, as shown in Figure 5, the CD8+ cell:granzyme B+ cell ratio was approximately 1.2. Only one of the seven HIVnE cases contained granzyme B-positive cells and that was a single cell in a perivascular infiltrate. Granzyme B–positive cells were absent in controls. Perforin IHC was not done in the cases with 0 to 1+ T-cell inflammation.

When postmortem cell shrinkage was absent or minimal, CD3+, CD8+ (Figure 5A), granzyme B+ (Figures 4 and 6B), and perforin+ cells formed direct cell-to-cell contact with neuronal cell body. There was no apparent correlation between morphological changes in neurons and the presence or absence of perineuronal T-cells. As depicted in Figures 5A and 6A and B, most neurons were histologically normal, without shrunken or eosinophilic perikarya, chromatin condensation or pyknosis, or formation of apoptotic bodies. Further studies to examine the relationship between CTLs and neuronal injury are pending in frozen tissue samples.

To confirm the presence of CTLs, we used double-label immunohistochemistry to determine whether granzyme B immunoreactive granules co-localized to CD8+ T cells. Because of the intensity of the CD8 reaction throughout the cytoplasm and the minute size of the granzyme B+ cytoplasmic granules, we only identified scattered cells that unequivocally displayed colabeling for these two antigens (Figures 6A–6C). Nevertheless, most of the labeled cells appeared to have an admixture of brown (CD8) and purple (granzyme B) and we did not detect cells that only were granzyme B immunoreactive.

Natural killer cells

To further support the hypothesis that the cytotoxic granules were of T-cell rather than of NK-cell origin, we were unable to identify any CD56+ NK cells in perivascular spaces or inflammatory nodules in either the HIVE or the HIVnE cases. Because normal brain is strongly immunoreactive for CD56, a key intercellular adhesion molecule in brain tissues (formerly called neural cell adhesion molecule), we cannot exclude the possibility that CD56+ cells were in brain parenchyma outside the nodular or perivascular collections of inflammatory cells.

Differential gene expression

Three of the five HIVE cases used for gene expression analysis contained HIVE inflammatory nodules with multinucleated giant cells and CD3+ T cell infiltrates in contralateral temporal lobe cortex. Multiple sections of cerebral cortex, including contralateral temporal lobe, were normal and without CD3+ T cells in the remaining two HIVE cases and all four HIVnE cases. We had sufficient RNA remaining from that extracted for gene expression studies to perform confirmatory reverse transcriptase (RT)-PCR for validation of gene expression changes in chemokine receptors (data not shown) and for RT-PCR to detect the presence or absence of viral RNA. RT-PCR for HIV-1 RNA identified viral sequences in 2 of 3 HIVE cases and 0 of 4 HIVnE cases.

The HG-U95 A Ver 2 array chip contains a total of 67 lymphocyte-associated genes, 8 of which are associated with cytotoxicity. Five of 10 significantly up-regulated genes were associated with cytotoxicity, including granzyme B and granzyme H (Table 3). Three of the *t*-tested genes were down-regulated; including IL-12A precursor, a CTL and NK maturation and proliferation gene (Gately *et al*, 1991; Gubler *et al*, 1991). Perforin gene expression was 4.0 ± 4.8-fold higher in HIVE than HIVnE but its high standard deviation gave it a *P* value of .055, which was slightly higher than our cutoff point.

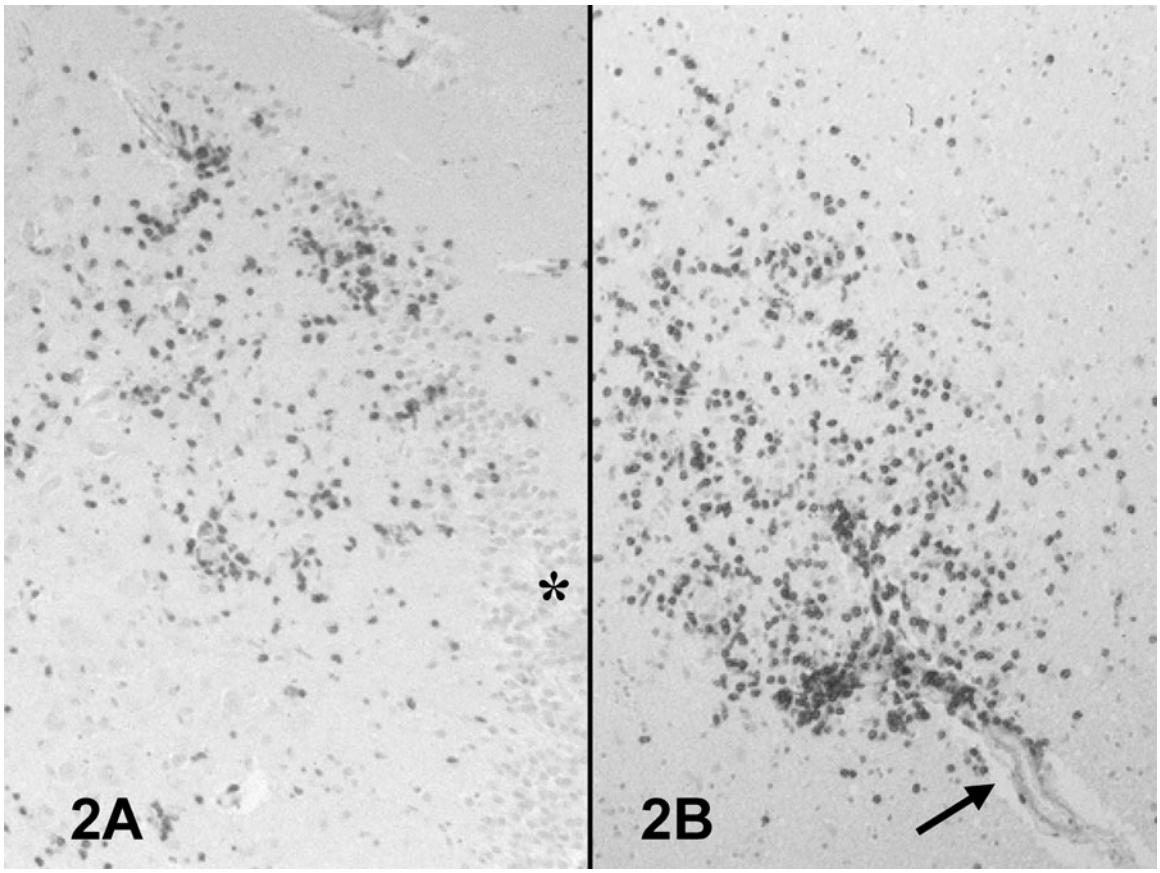

Figure 2 HIVE. Large numbers of CD3+ T lymphocytes accumulate within the CA4 region of the hippocampus (**A**) and temporal lobe white matter (**B**) in a patient with HIVE. Asterix indicates the dentate gryus in **A**; arrow indicates a small blood vessel in **B**. Hematoxylin counterstain; original magnification ×100.

These results validate the immunohistochemical studies showing that cytotoxic cells accumulate in brains of HIVE. Because whole-tissue samples were used, the cell of origin of the cytotoxic-associated genes cannot be identified and could include CD4 T cells or NK cells. Furthermore, the presence of opportunistic infections in two of the five HIVE cases may have participated in T-cell recruitment, although we consider this unlikely because they were healed lesions, remote from the temporal lobe sampled for RNA extraction.

Discussion

HIV-1–specific cytotoxic T cells are present in sera of AIDS patients (Walker *et al*, 1987) and play a key role in viral suppression during the acute and chronic phase of infection (Gamberg *et al*, 1999; Brander and Riviere, 2002). Their daily turnover increases more than six-fold compared with HIV-1–uninfected patients and their absolute numbers remain elevated even when the CD4 count falls to less than 200 (Sachsenberg *et al*, 1998). Animal models, including simian immunodeficiency virus (SIV) infection and a severely compromised immunodeficient (SCID) mouse model for HIV-1, duplicate the viral suppressive activities of the CD8+ T cells (Adleman and Wofsy, 1993; Kaur *et al*, 2000; Schmitz *et al*, 1999). The antiviral activity of the CD8+ cells is based on their perforin/granzyme death mechanisms because the relative fraction of perforin-expressing CD8+ T cells inversely correlates with CD4+ T cell levels in peripheral blood (Heintel *et al*, 2002). Despite a brisk CTL response to HIV-1 and genetic diversity to almost the entire spectrum of viral proteins (Brander and Riviere, 2002), CTLs eventually fail to suppress viral reproduction.

Cytotoxic T lymphocytes rapidly accumulate in the central nervous system in animal models of HIV-1. In macaques, CTLs infiltrate the cerebrospinal fluid (CSF) and brain within 1 week of systemic SIV infection (von Herrath *et al*, 1995). In SCID mice, HIV-1–specific CTLs infiltrate brain within 1 week after intrabrain injection of HIV-1–infected macrophages (Poluektova *et al*, 2002). HIV-1–specific CTLs effectively suppress microglial cell infection of mixed glial/neuronal cell cultures and do so in the apparent absence of cell lysis (Lokensgard *et al*, 1999). T-cell entry could be via the intraparenchymal vessels

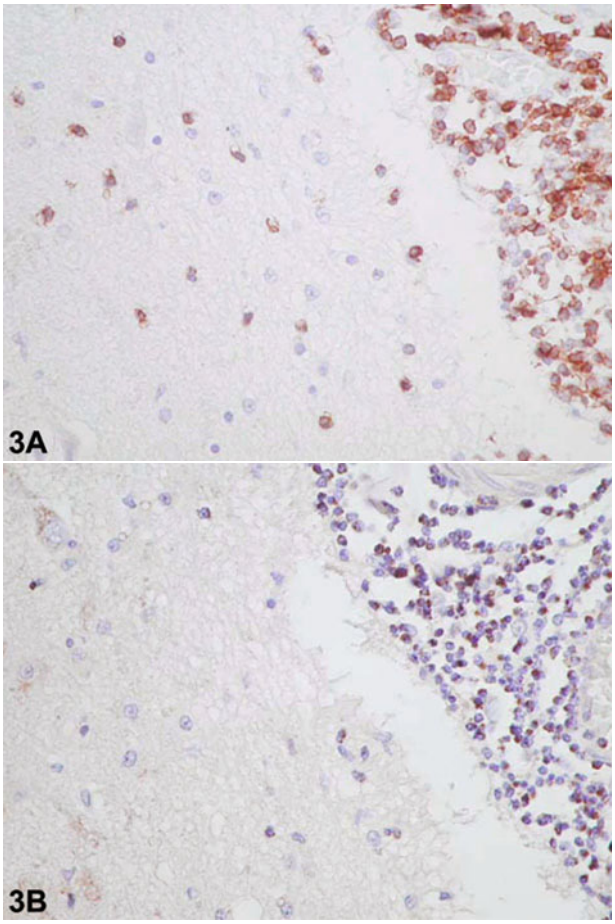

Figure 3 A dense perivascular lymphocytic infiltrate in the same CA4 region shown in Figure 2A is composed primarily of CD8+ T cells (**A**). On serial section, many also are granzyme B+ (**B**). Hematoxylin counterstain; original magnification ×400.

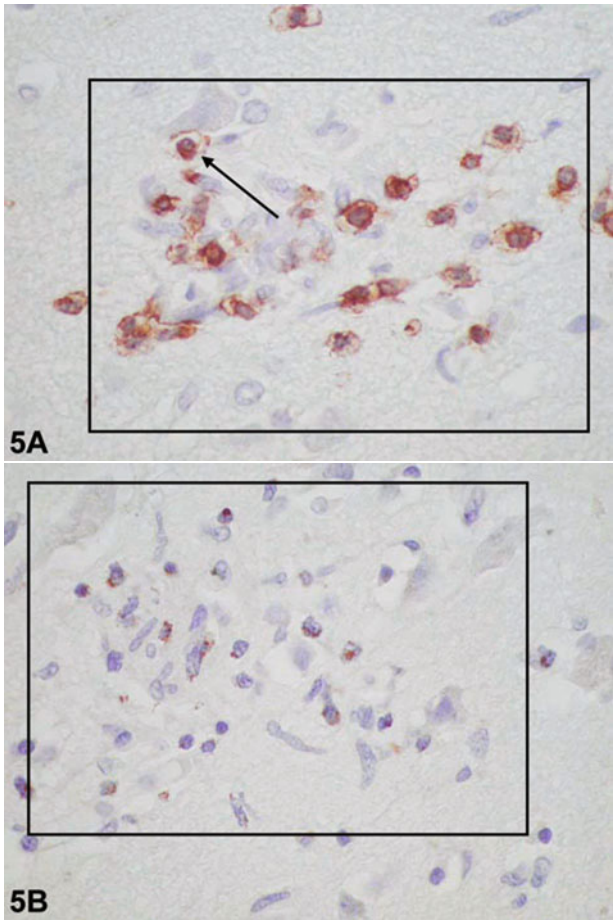

Figure 5 Serial sections through an inflammatory nodules of HIVE shows a similar number of (**A**) CD8+ T cells ($n = 16$) and (**B**) granzyme B+ cells ($n = 14$) within the indicated square. The arrow in **A** points to a CD8+ T lymphocyte in direct contact with a neuronal cell body. Hematoxylin counterstain, original magnification ×600.

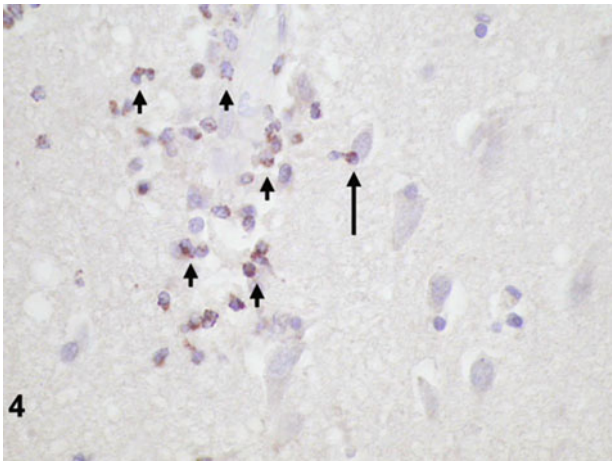

Figure 4 An inflammatory nodule in the cortex of a HIVE patient contains numerous inflammatory cells that contain minute granzyme B+ cytoplasmic granules, some of which are indicated with arrows. The long arrow shows a granzyme B+ cell in direct contact with a neuronal cell body. Hematoxylin counterstain, original magnification ×600.

or the CSF because these cells are increased in choroid plexus and perivascular spaces in AIDS brains (Falangola *et al*, 1995; Katsetos *et al*, 1999) and preferentially enter the choroid plexus after peripheral immune activation (Petito and Adkins, 2005).

This present study shows that CTLs accumulate in HIVE brains as well as in its animal models. Although specific information concerning therapy was absent in many of our cases, all had AIDS for at least several years and died during or subsequent to 2001. Most were under a physician's care and thus likely to have been exposed to HAART. The number of CTLs directly correlated with productive viral infection and HIVE. Although they appeared to be fewer in number than CD3+ and CD8+ T cells, it is probable that their numbers were underrepresented due to the relatively small size of the cytoplasmic granules compared with the diffuse CD3+ and CD8+ membrane staining of the entire T cell. CD8+ T cells and CTLs were present in

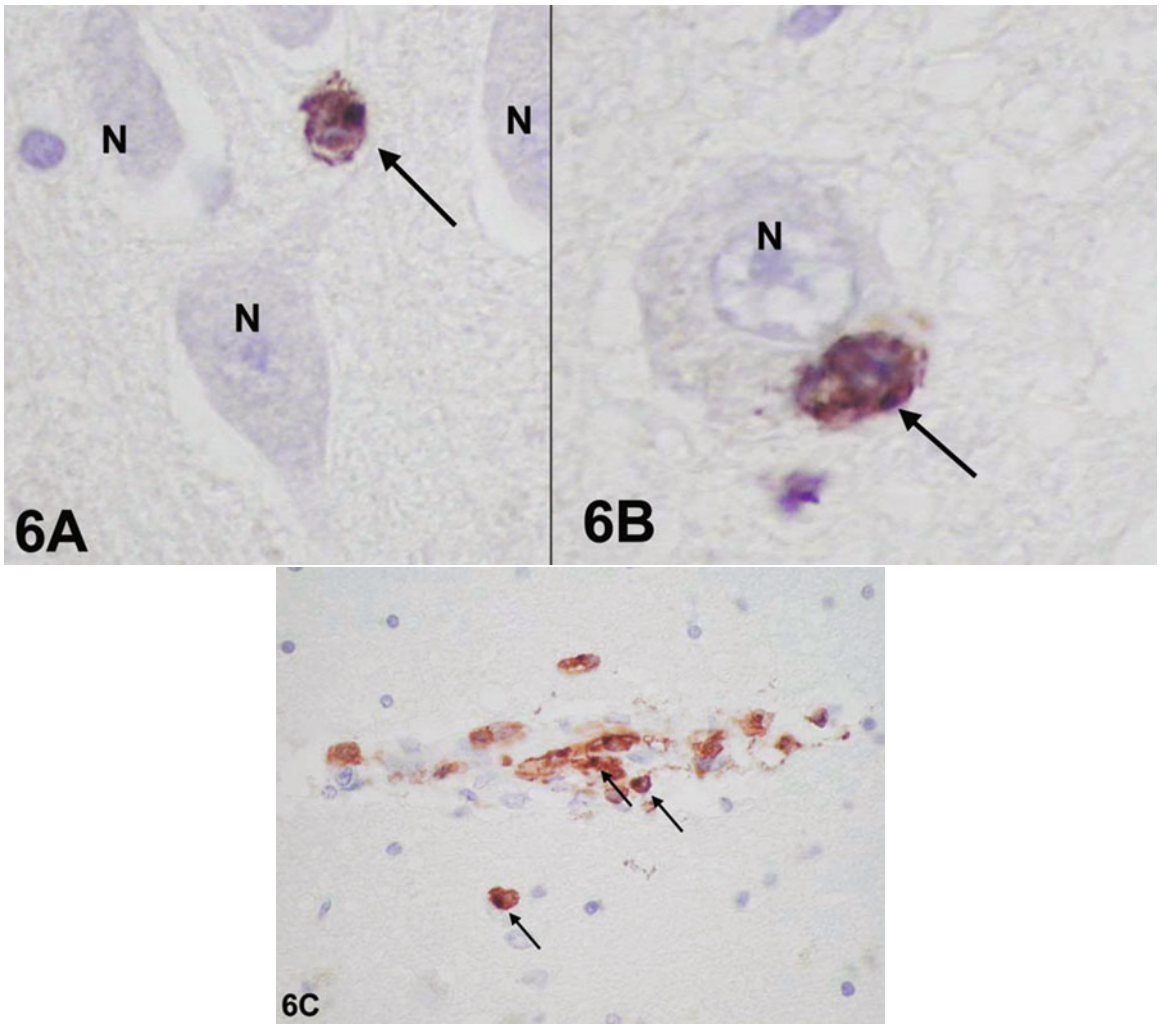

Figure 6 Double-label immunohistochemistry for CD8 (*brown*) and granzyme B (*purple*) shows CD8+ T lymphocytes that contain granzyme B+ cytoplasmic granules (*arrows*). These cells are shown in gray matter (**A**), in direct cell-to-cell contact with neurons (**B**), and in perivascular spaces and brain parenchyma (**C**). N: neuronal nuclei. Hematoxylin counterstain; original magnification ×600.

perivascular spaces, inflammatory nodules and brain parenchyma. Most of HIVE cases had CTLs, as well as CD8+ T cells, in direct contact with neuronal cell bodies, a finding we previously reported for the CD8+ cells (Petito *et al*, 2002). In support of the presence of CTLs in HIVE brains, two CTL-associated genes were up-regulated in HIVE cortex compared with HIVnE cortex (granzyme B precursor and granzyme H). Perforin gene expression also was up-regulated in HIVE versus HIVnE, but its P value of $<.055$ did not quite reach significance due to a high standard deviation (SD) among the HIVE cases. Because the distribution of HIVE lesions are nodular rather than diffuse, and the apparent number of perforin+ cells was lower than that of the granzyme B+ cells, the high SD is not unexpected.

We did not find NK cells in our HIVE brains, which is in contrast to the SIVE model in which NK cells as well as CD8+ T cells have been described (Mankowski *et al*, 2002). In the primate model, the percentage of NK cells and CD8+ T cells was high in three animals with severe SIVE and mild in two with mild SIVE as well as one animal without encephalitis (Mankowski *et al*, 2002). The absence of NK cells in our HIVE brains may be due to less sensitive detection methods because limited markers for NK cells are available for paraffin sections. Alternatively, intrinsic differences between our AIDS patients and the SIVE model could be responsible for the absence of NK cells in HIVE. The AIDS patients in our study had a prolonged HIV-1 infection and were exposed to HAART for months or years. In contrast, the SIVE animals had accelerated infection without treatment exposure. Although HAART is associated with restoration of NK cell numbers and activities (Sondergaard *et al*, 1999; Weber *et al*, 2000), the beneficial effects on NK cells is lost once HAART no longer suppresses viral load. Thus, in our end-stage AIDS patients, it

Table 3 Differentially expressed lymphocyte-associated genes in HIVE versus HIVnE cortex

Fold	*GeneBank ID*	*Cytotoxicity*	*Gene name*	*Gene function*
6.63	AL109669		IL-16	Lymphocyte chemoattractant factor
4.781	L41268	X	Killer cell IG-like receptor	Killer cell immunoglobulin-like receptor
3.876	M17016	X	Serine protease-like protein	Granzyme B precursor, cytotoxicity
3.625	Y00636		CD58 antigen	Lymphocyte function-associated antigen 3
3.433	M25280		Selectin L	Lymphocyte adhesion molecule 1
2.443	AF011333		Lymphocyte antigen 75	Antigen endocytosis by dendritic cells (Jiang *et al*, 1995)
2.338	M81141		MHC class II, DQ beta 1	MHC, class II
2.153	M57888	X	CTL-associated serine esterase 1	Granzyme H; cytotoxicity
2.045	×61072	X	TCR V alpha 14	T-cell receptor (CD8+ tumor-infiltrating lymphocyte)
2.017	L25851	X	Integrin, alpha E (CD103)	Allows CD8+ T cells to bind to epithelial-specific E-cadherin (Feng *et al*, 2002)
0.476	AL050135		Regulatory factor X, 5	Influences HLA class II expression
0.41	AJ012008		Lymphocyte antigen 6 complex	Lymphocyte antigen 6 complex, locus G6C
0.375	M65291	X	IL 12A precursor	NK cell stimulatory factor; CTL maturation factor
0.226	M69199		G0S2 protein	Putative lymphocyte G0/G1 switch gene

Abbreviations: IL: interleukin; IG: immunoglobulin; MHC: major histocompatibility complex; CTL: cytotoxic T lymphocytes.

is likely that systemic NK-cell activity was reduced as well.

Because the presence and number of parenchymal T cells and CTLs was almost specific for HIVE, we suggest that their presence in the brain is a biomarker for productive HIV-1 infection in the absence of opportunistic infections or lymphoma. All of the HIVE cases contained numerous CD3+, CD8+, and CTL-positive T cells in the brain, whereas only one of the HIVnE and none of the controls contained these cells. As discussed, it is likely that the one HIVnE case with moderate numbers of white matter T cells had productive HIV-1 infection in brain regions not sampled for microscopic examination.

In peripheral lymphoid organs, cytotoxic T lymphocytes colocalize to productively infected cells or apoptotic cells (Brodie *et al*, 2000; Hosmalin *et al*, 2001). We suggest that productive brain infection is required for T-cell persistence and infiltration in brain parenchyma because parenchymal T cells, including the CTLs expressing perforin and granzyme B, were almost always confined to cases with HIVE. Indeed, it is likely that parenchymal CD3+ and CD8+ T cells and CTLs may be regarded as biomarkers for HIVE. The perineuronal localization of T cells and CTLs could be related to latent HIV-1 infection of neurons as described previously (Torres-Munoz *et al*, 2001; Trillo-Pazos *et al*, 2003). Previously, we showed that CD3+ T cells were specific for HIVE in hippocampal subregions (Petito *et al*, 2002). The current study modifies this observation in two ways. First, it shows that accumulation of parenchymal CD8+ T cells as well as CD3+ T cells is sensitive for, and may be specific for, HIVE. Second, it suggests that T-cell accumulation is widely distributed in HIVE brains and not necessarily confined to locally productive infection. This widespread distribution was most apparent in the white matter, a phenomenon that may be due to the intrinsic ability of activated T cells to rapidly migrate through white matter tracks (Kruse *et al*, 1994).

Cytotoxic T lymphocytes kill neurons and glia by releasing their effector molecules, perforin, and granzyme, a rapid process of cytolysis initiated by perforation of the target cell molecule by CTLs perforin-positive tubular protrusion (Lichtenheld *et al*, 1988). CTL killing also develops more slowly when the CTL Fas ligand binds with the target cell's Fas cell death receptor. Both processes require antigen presentation by major histocompatibility complex (MHC) class I or II on the target cell for subsequent binding to the T-cell receptor (Rall *et al*, 1995; Russel and Levy, 2002; Neumann *et al*, 2002; Giuliani *et al*, 2003). An alternative ligand-receptor interaction develops under stress conditions or with viral infections with the induction of target cell MHC class I chain-related genes, MICA and MICB, which interact with a T-cell receptor, NKG2D (see Collins, 2004, for review). MICA/B-NKG2D forms a sufficiently stable interaction to permit perforin/granzyme-mediated cell lysis (Somersalo *et al*, 2004).

Earlier studies have not detected neuronal MHC class I expression or Fas in AIDS brains (Achim *et al*, 1991; Kennedy and Gairns, 1992; Elovaara *et al*, 1998) and MICA/B molecules have not been examined. For several reasons, however, it is likely that neuronal MHCs or MICA/B expression might be found with newer techniques or antisera. Interferon-γ is increased in AIDS brains (Tyor *et al*, 1992). The significance of this observation lies in INF-γ's ability to up-regulate or induce neuronal MHC I γ *in vitro*, allowing CTLs to kill neurons by the perforin/granzyme B-mediated pathways (Rensing-Ehl *et al*, 1996) or the Fas/Fas Lγ pathway (Medana *et al*, 2000). Furthermore, CTL-mediated neuronal death via perforin/granzyme or Fas/FasL pathways occurs with viral infections and autoimmune disorders *in vivo* (Gogate *et al*, 1996; Medana *et al*, 2000; Neumann *et al*, 2002; Bien *et al*, 2002; Hoftberger

et al, 2004). Lastly, there is recent evidence that activated (Guilliani *et al*, 2003) or perforin/granzyme-expressing T cells (Nitsch *et al*, 2004) can directly contact and lyse MHC class I-negative neurons in multiple sclerosis.

T cells can damage brain by pathways other than CTL-mediated direct cell killing. These cells secrete neurotoxic cytokines such as tumor necrosis factor (TNF)-α . Second, there is some evidence that CD8+ as well as CD4+ T cells are infected by HIV-1. McBreen *et al* (2001) detected HIV sequences more frequently and at higher levels in naive (CD45RA+) versus memory (CD45RO+) CD3+CD8+ T cells in 9 of 16 infected patients. The higher frequency of CD4+ activated CD8+ T cells (Imlach *et al*, 2001) would render these cells more susceptible to HIV infection. Thus, HIV-1–infected CTLs could introduce, or more likely, reintroduce virus into the CNS. T cells also could reactivate latent neuronal infection *in vivo*, as they do to astrocytes *in vitro*, by changing a restricted HIV infection into a productive infection upon secretion of their proinflammatory cytokines (Tornatore *et al*, 1991). Lastly, perineuronal T cells, as well as microglia, express the requisite CD4 receptor for HIV entry and thus could participate in transreceptor-mediated viral entry into chemokine receptor-positive, CD4-negative neurons. Such transreceptor viral entry takes place in astrocytes *in vitro* (Speck *et al*, 1999).

Materials and methods

For immunohistochemistry studies, we selected autopsy cases with AIDS and HIVE ($n = 8$), AIDS and normal brains (HIVnE) ($n = 8$), and age-matched controls with neither clinical nor pathological evidence of HIV-1 infection ($n = 10$). For the microarray studies, we selected a separate series of HIVE and HIVnE cases, four per group, with fresh-frozen brain samples taken at the time of autopsy. For the purposes of this study, we used the characteristic multinucleated giant cell as a marker for HIVE and for active viral production (Budka *et al*, 1991). Material was used with University of Miami Institutional Review Board approval. Some AIDS cases were generously donated by the National NeuroAIDS Tissue Consortium, University of Texas at Galveston (MH59656 and MH59724). Some controls were donated by the National Institute of Child Health and Development Brain and Tissue Bank for Developmental Disorders, University of Miami (NO1-HD43383 and NO1-HD43368). Brains had been fixed in 10% buffered formalin for 10 to 14 days prior to brain dissection and submission of multiple sections for paraffin embedding and histological examination.

Immunohistochemistry

We cut 5μ thick serial sections from paraffin blocks of hippocampus-temporal lobe and basal ganglia because these two areas usually are most affected by HIVE. After deparaffinization, we steamed the sections in citrate buffer for 20 min and then sequentially incubated them in normal serum, primary antibody, secondary antibody, avidin-biotin complex, and H_2O_2 plus 3, 3′-diaminobenzidine. Primary monoclonal antibodies (mAbs) on serial sections included those directed against CD56 for NK cells and a subset of activated T cells (Novocastra, Newcastle upon Tyne, UK; 1:50 dilution); perforin (Kamiya Biomedical, Seattle, WA; 1:1000 dilution); CD3 for T cells (Santa Cruz Biotechnology, Santa Cruz, CA; 1:300 dilution); granzyme B (Cell Sciences, Canton, MA; 1:80 dilution); and CD8 (Abcam; 1:80 dilution). For the double-labeled immunohistochemistry, slides were steamed in citrate buffer and sequentially incubated with anti-CD8 mAb overnight at 4°C, biotinylated secondary antibody for 1 h, ABC complex, H_2O_2 and 3, 3′-diaminobenzidine (to give a brown color), immediately followed by anti-granzyme B mAb for 1 h at 27°C, biotynlated secondary antibody, ABC complex, H_2O_2, and VIP (Vector labs) (to give a purple color). Positive controls included lymphocytes within intravascular components on the tissue section as well as those in mouse intestine and human tonsil.

Quantification

We first surveyed the CD3+ T-cell distribution in basal ganglia and hippocampus by determining their numbers in perivascular spaces and brain parenchyma with the following 0–3+ grading scale. Perivascular T cells: 0: none; 1+: occasional T cells, <1 layer thick, around some vessels; 2+:>1 layer of T cells around several small or large blood vessels; 3+ > 1 layer of T cells around many blood vessels. Parenchymal T cells: 0: none or <2 per slide; 1+: occasional T cells; 2+: many T cells diffusely infiltrating brain; 3+: focal collections of T cells, with or without diffuse T-cell infiltration. Because the distribution was similar in basal ganglia and hippocampus, and because the latter section provided information concerning cortical and white matter disease, the following studies were confined to the hippocampal sections. We used the same 0–3+ grading scale to evaluate the number of perivascular CD8+ T cells in temporal lobe cortex, white matter and CA4 region of the hippocampus. We determined the number of parenchymal CD8+ T cells per 100× microscopic field in the same three regions. We evaluated CD56+, granzyme B+, and perforin+ cells as present or absence because their numbers were low and because the minute small size of the immunoreactive granules of perforin and granzyme required examination at a magnification of 400×.

Data reduction and analysis

We determine significant differences in age and PMI with analysis of variance (ANOVA) and the Bonferroni multiple comparison test for parametric data in the three groups of the IHC study and the unpaired *t* test for parametric data in the two groups of the gene

expression study. We used the Kruskal-Wallis test for nonparametric data to determine whether there were significant differences in perivascular and parenchymal CD3+ T cells among groups in the IHC study.

Differential CTL gene expression and data analysis
After removing the leptomeninges, we minced fresh frozen temporal lobe cortex, extracted total RNA, and then amplified and labeled mRNA. We evaluated its quality with the Agilent Bioanalyzer microchip electropherograms, detection of housekeeping genes, and by adequate hybridization with the Affymetrix (Santa Clara, CA) test chips. Biotin-labeled aRNA was hybridized onto oligonucleotide gene array chips HG-U95 A Ver 2 (Affymetrix, Santa Clara, CA), scanned with Affymetrix scanning platforms, and resultant scanned data imported into the GeneSpring software version 6.1 (Silicon Genetics, Redwood, CA) for analysis as previously described (Torres-Muñoz *et al*, 2004b) Each microarray was normalized per chip to the 50th percentile and per gene to the median values, using included housekeeping and common gene sequences on the chip. We used the signal data in ratio mode interpretation and selected those genes with a fold expression difference of |2|-fold. We determined their statistically significance with the parametric ANOVA post hoc test, with $P < .05$ for significance, using log of ratio mode interpretation. The resultant lists of *t*-tested genes with |2|-fold differences were grouped using Gene Ontology and GeneMAPP pathways. For this study, we looked at differential expression for all sequences identified as T lymphocyte genes and their subgroup identified as cytotoxicity genes, and compared gene expression in HIVE versus HIVnE; cases. Microarray results were confirmed for four differentially expressed genes by quantitative RT-PCR as described previously (Torres-Muñoz *et al*, 2004a), normalizing the value of each experimental gene value to value of the housekeeping gene GAPDH in the same sample, and expressing results as % of HIV-1–negative controls (data not shown).

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