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Human immunodeficiency virus infection inhibits granulocyte-macrophage colony-stimulating factor–induced microglial proliferation

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It is well known that infection by the human immunodeficiency virus (HIV) dysregulates cell physiology, but little information is available on the consequences of HIV infection in primary macrophages and microglia. The authors examined the relationship between cell proliferation and HIV infection in primary cultures of microglia and in human central nervous system (CNS). In cultures infected with HIV (ADA and BaL), granulocyte-macrophage colony-stimulating factor (GM-CSF)–mediated cell proliferation was reduced in productively infected (p24+) cells as compared to p24− cells. The reduction was observed with both Ki67 and BrdU labeling, suggesting a G1/S block. The reduction was insignificant when microglia were infected with a Vpr− mutant virus. In human CNS, proliferating (Ki67+) cells were rare but were increased in the HIV+ and HIV encephalitis (HIVE) groups compared to the HIV− group. A positive correlation between GM-CSF immunoreactivity and Ki67 counts, implicating GM-CSF as a growth factor in human CNS was found. The relationship between total macrophage (CD68+) proliferation and infected macrophage (p24+) proliferation was assessed in HIVE by double labeling. Whereas 1.2% of total CD68+ cells were Ki67+, only 0.5% of HIV p24+ cells were Ki67+ ($P < .05$). Furthermore, staining for CD45RB (as opposed to CD68) facilitated the identification of Ki67+ microglia, indicating that CD68 could underestimate proliferating microglia. The authors conclude that although there is increased expression of GM-CSF and increased cell proliferation in the CNS of HIV-seropositive individuals, cell proliferation in the productively infected population is actually suppressed. These data suggest that there might be a viral gain in the suppressed host cell proliferation. *Journal of NeuroVirology* (2007) **13,** 536–548.

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Introduction

Central nervous system (CNS) human immunodeficiency virus (HIV) infection can manifest as HIV-associated dementia (HAD). HIV infection of the brain produces characteristic histopathologic features such as formation of multinucleated giant cells and microglial nodules (referred to as HIV encephalitis [HIVE]). Macrophages and microglia represent the majority of productively infected cells in HIVE, and infected and activated brain macrophages have been implicated as the prime instigators of neuronal damage in HAD (Dickson and Lee, 1997;

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Gonzalez-Scarano and Baltuch, 1999; Kolson *et al*, 1998; Lipton and Gendelman, 1995). The importance of tissue macrophages as persistent viral reservoirs has also been emphasized in the post-HAART (highly active antiretroviral therapy) era (Collman *et al*, 2003).

In normal adult CNS, there is limited turnover of cells. Although neurons are considered terminally differentiated, attempts at reentering the cell cycle have been demonstrated and correlated with neuronal degeneration (Jordan-Sciutto *et al*, 2000, 2002; Vincent *et al*, 1996). Moreover, different types of glia (astrocytes, oligodendrocytes, and microglia), as well as endothelial cells and inflammatory cells, can undergo proliferation in injured CNS. Microglial proliferation has been shown in numerous occasions in humans as well as in experimental models of CNS injury (Graeber *et al*, 1998; Matsumoto *et al*, 1992; Norton, 1999; Postler *et al*, 1997; Raivich *et al*, 1991; Schonrock *et al*, 1998). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) are principal microglial growth factors and their expression (along with their cognate receptor expression) is up-regulated in injured or diseased CNS (Giulian and Ingeman, 1988; Lee *et al*, 1994; Raivich *et al*, 1991).

A lentivirus such as HIV is unique in that it can infect nondividing cells. This is different from other retroviruses, which require host cell mitosis for nuclear transport of the viral nucleic acids (Stevenson, 2000). Although controversy still exists as to the requirement of cell proliferation for HIV replication in macrophages (Kootstra *et al*, 2000; Kootstra and Schuitemaker, 1998; Schuitemaker *et al*, 1994), results from most laboratories seem to indicate that cell proliferation is not necessary for HIV infection of macrophages or microglia (Heinzinger *et al*, 1994; Schmidtmayerova *et al*, 1997; Stevenson, 2000). This notion is in part supported by the observation that the amount of HIV production from proliferating and nonproliferating microglial cultures is similar (Si *et al*, 2002a). Cell proliferation is a complex and highly coordinated cellular process that is often targeted by invading pathogens such as viruses. Notably, host cell cycle block has been consistently documented following HIV infection of a wide array of cells including T cells, T-cell lines, and tumor cells (Scholzen and Gerdes, 2000; Sherman *et al*, 2002). The cell cycle arrest is reportedly at G2 and the HIV accessory protein Vpr is found to be both necessary and sufficient in most cells (Amini *et al*, 2004; Chowdhury *et al*, 2003; He *et al*, 1995; Planelles *et al*, 1996). In relevance to the CNS, we have recently shown that primary human astrocytes that are productively infected by VSVg-*env*–pseudotyped HIV also show a cell cycle block (Cosenza-Nashat *et al*, 2006b). Although macrophages represent important tissue reservoirs for HIV infection, the details of cell cycle block in infected macrophages and in tissues such as the CNS are not known. In the current study, we sought to delineate the impact of HIV infection on macrophage/microglial proliferation in primary cultures of microglia and in human CNS.

Results

Studies of human microglia in vitro: HIV infection decreases Ki67 and BrdU labeling of microglia

Microglia were exposed to GM-CSF and two M-tropic strains of HIV, ADA and BaL (Vpr+ and Vpr−), and were harvested at 7 or 10 days post infection, the earliest time points that allow detection of productive infection in microglia (Lee *et al*, 1993; Si *et al*, 2002b, 2004). In these cultures, the percent of p24+ cells ranged from 4% to12% of total microglia, but the addition of GM-CSF did not significantly alter the infection rate (Figure 1A), consistent with our previous observations (Si *et al*, 2002a). We then analyzed the percent of Ki67+ cells among uninfected (p24−) and infected (24+) populations. In cultures not treated with GM-CSF, the proliferation rate was very low and no significant differences were found between p24+ and p24− populations (Figure 1B). In cultures treated with GM-CSF, the proliferation rate increased modestly. Although the optimal time point for detection of microglial proliferation (∼10%) is 3 to 4 days after GM-CSF, for simultaneous detection of infection, the cells were harvested at 1 week, which resulted in a lower Ki67 labeling index (∼3%; Figure 1C) (Lee *et al*, 1994; Suh *et al*, 2005). In these cultures, Ki67 labeling was lower in the p24+ cell population compared to the p24− population (Figure 1C). Analysis with an S-phase marker BrdU also showed lower BrdU labeling indices in p24+ cells compared to p24− cells (Figure 1D). However, in cultures infected with a Vpr mutant HIV (BaL Vpr−), the difference in proliferation between the two populations became insignificant (Figure 1C and D).

Figure 2 illustrates examples of double labeling of microglial cultures. All panels show GM-CSF–treated cultures except Figure 2A. Proliferation of microglia increased with GM-CSF as determined with Ki67 (Figure 2B) or BrdU (Figure 2C) immunocytochemistry. In cultures infected with HIV_{ADA}, double labeling for BrdU and p24 showed nuclear BrdU labeling in noninfected cells (Figure 2D). Similarly, in cultures infected with ADA, double labeling for Ki67 and p24 showed nuclear Ki67 labeling in the p24− cell population (Figure 2E). Infected microglial cells in these cultures were very often multinucleated and were positive for cytoplasmic p24 immunoreactivity (Figure 2E). Cultures infected with BaL Vpr+ were compared with those infected with BaL Vpr− by either BrdU labeling (Figure 2F and G) or Ki67 labeling (Figure 2H and I). In cultures infected with BaL Vpr+, proliferating cells tended to be noninfected (Figure 2F and H), whereas in cultures infected with BaL Vpr−, many proliferating cells were also infected (p24+; Figure 2G and I). Therefore, the

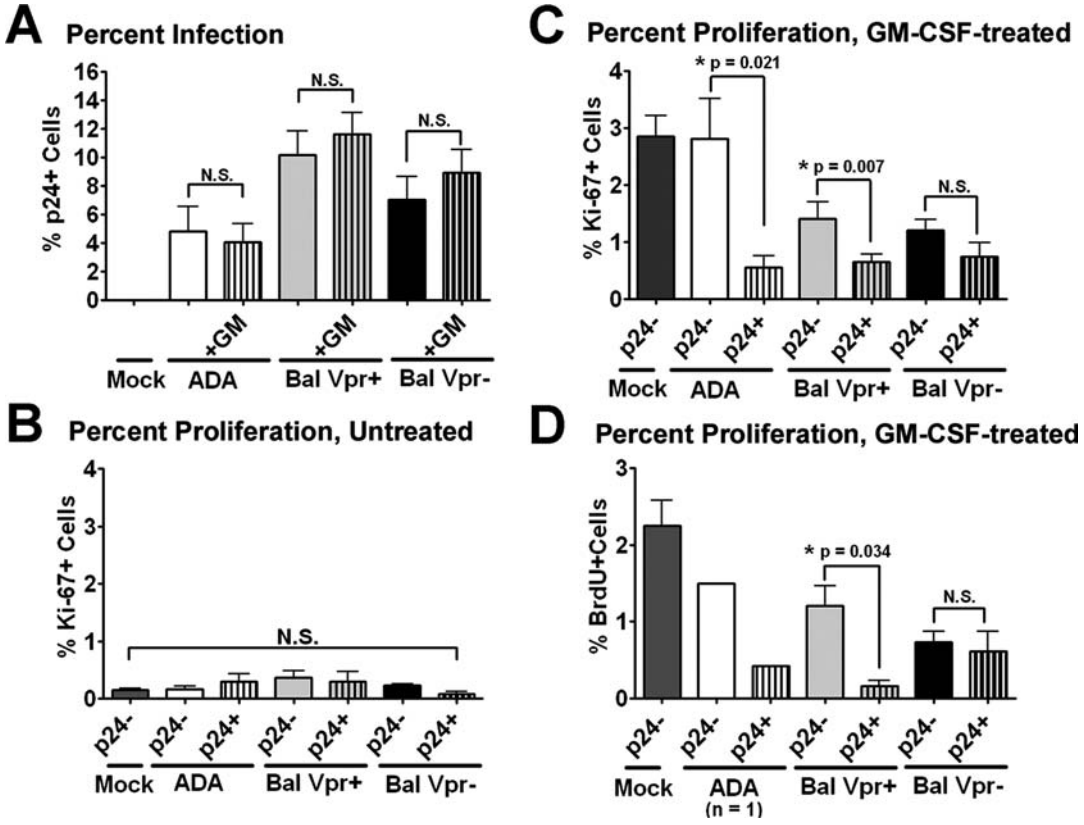


Figure 1 Quantitative analysis of microglial proliferation and HIV infection *in vitro*. In order to determine the relationship between cell proliferation and HIV infection, microglial cultures were exposed to GM-CSF and HIV (ADA, BaL Vpr+, BaL Vpr−) or mock supernatant and, were double-labeled for a proliferation marker (Ki67 or BrdU) and p24 *gag* antigen as described in the Materials and Methods. Cells in the entire well were counted and data were expressed as the percent positive cells. Data are pooled from three different cases of microglia, each consisting of triplicate wells, except the BrdU labeling of ADA culture ($n = 1$). For photographic examples of double-labeled cells, see Figure 2. (**A**) The level of infection varied for the three different viruses, but no difference was noted in the infection rate with or without GM-CSF. (**B**) Proliferation of untreated microglia was minimal with no significant difference among groups (mock or different virus exposure). (**C**) Ki67 labeling in GM-CSF–treated and HIV-infected cultures was analyzed in p24+ and p24− populations separately. The percent Ki67 labeling was lower in p24+ cells compared to the p24− microglia population. However, the difference was small and insignificant (N.S.) in Vpr− cultures. (**D**) BrdU labeling showed essentially the same findings, with the proliferation rate in p24+ cells being lower than that in p24− cells. No difference was found in cultures infected with the Vpr− virus. Statistics could not be performed for BrdU in ADA culture, as only one out of three cases showed a high enough level of infection at the time point studied.

results from *in vitro* microglial experiments demonstrate that HIV infection limits cell proliferation and that Vpr may play a role in the observed cell cycle block.

Studies of human CNS in vivo: proliferating (Ki67+) cells are increased in the brains of HIV+ individuals

Because the *in vitro* findings suggested that HIV infection reduces microglial proliferation, we next examined human CNS tissue to determine whether HIV-infected cells (macrophages and microglia) also show a block in cell proliferation. We first examined total proliferating cells by single-label Ki67 immunohistochemistry on postmortem paraffin-embedded brain sections from three patient groups (HIV−, HIV+ but no HIVE, and HIVE), as described in Materials and Methods. By morphology, the Ki67+ cells resembled glial cells, macrophages, and endothelial cells (Figure 3A to C and insets). Ki67+ cells were counted in the entire white matter area of each section single-labeled for Ki67 and data are expressed as the number of Ki67+ cells per mm^2. Although generally rare, Ki67+ cells were increased in sections of HIV+ and HIVE compared to HIV− brains (Figure 3A to C). The results shown in Figure 3D demonstrate that proliferating cells were rare with average Ki67 counts ranging from ~1 to ~5 per mm^2 brain area. The average number of Ki67+ cells was higher in HIV+ and HIVE compared to the HIV− group, with large case variability, especially for the HIV+ group (Figure 3D). When the Ki67 cells were further analyzed with respect to their location, both vessel-associated and parenchymal counts increased in the HIV+ and HIVE groups compared to the HIV− group (Figure 3D). Analysis of data with one-way analysis of variance (ANOVA) demonstrated that vessel-associated (as well as total) but not parenchymal Ki67 counts were significantly

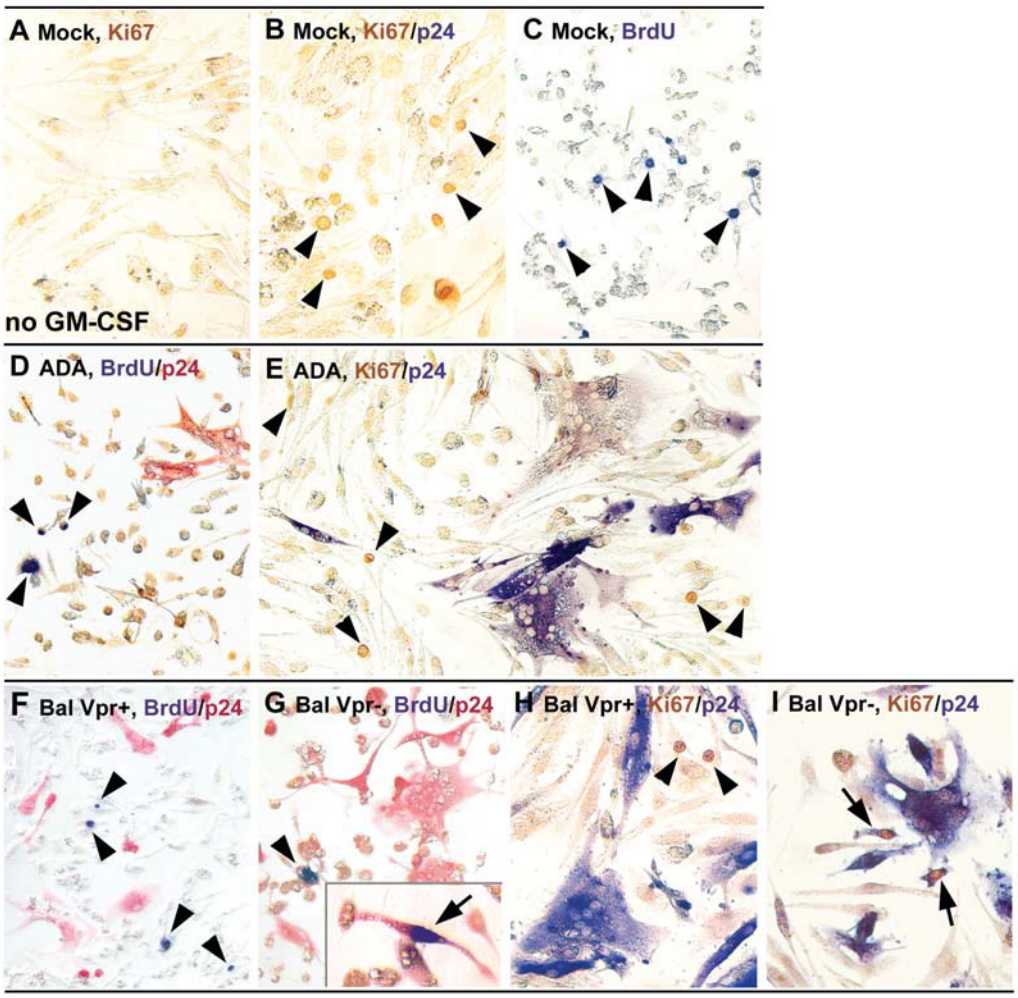
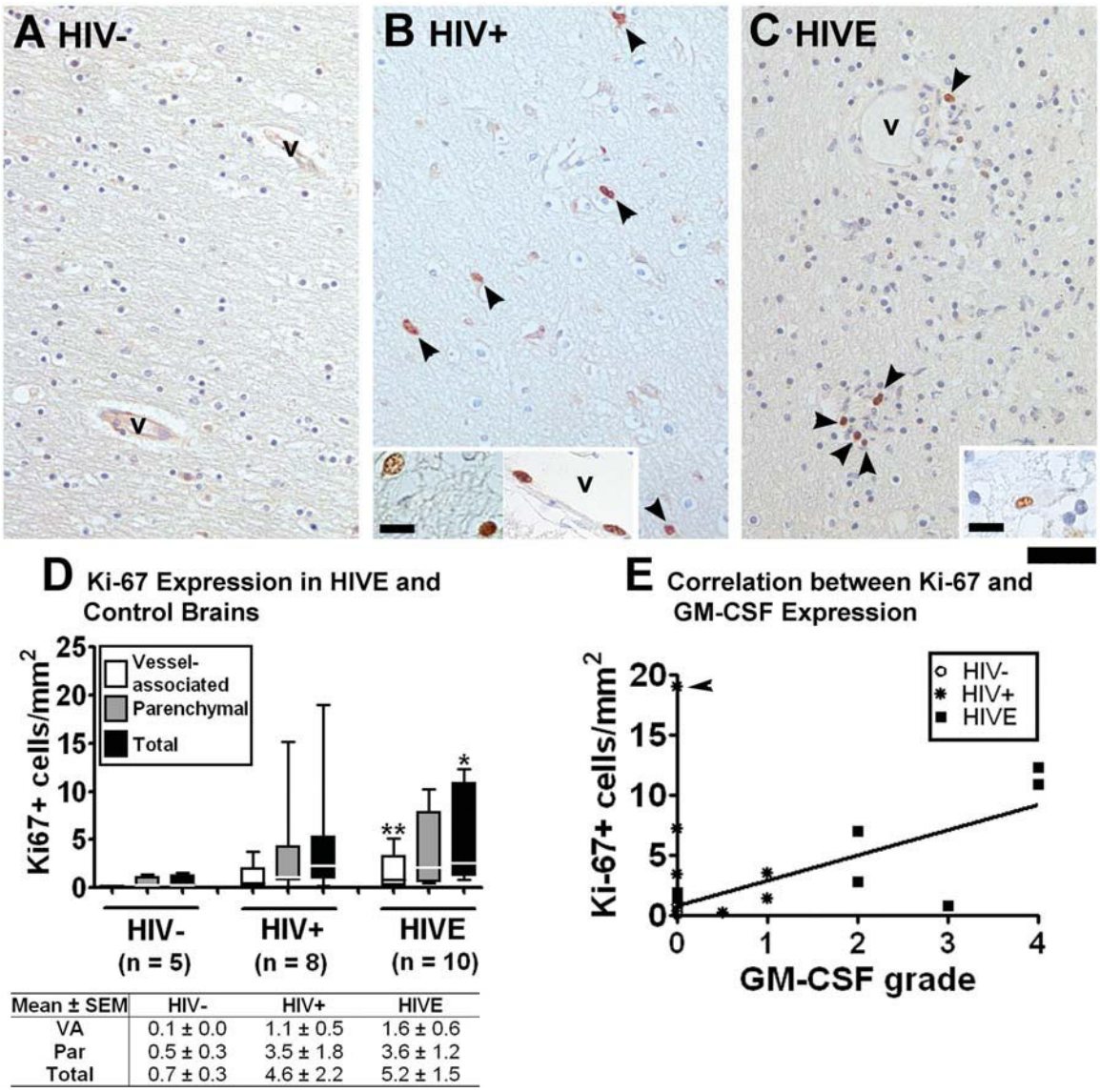


Figure 2 Ki67 and BrdU labeling in HIV-infected microglia *in vitro*. Cultures exposed to GM-CSF and HIV as described in Figure 1 legend were double-labeled for proliferation (Ki67 or BrdU) and infection (p24) markers. Figure labels match the color of chromogen used for each antigen. Cultures shown were treated with GM-CSF except for **A** (untreated: no GM-CSF). (**A–C**) Mock-infected cultures double-labeled for p24 and proliferation markers. Ki67+ or BrdU+ nuclei (*arrowheads*) were increased in GM-CSF–treated cultures. No p24 stain was detected in mock-infected cultures. (**D, E**) Microglial cultures infected with ADA and treated with GM-CSF show the proliferation marker (BrdU+ blue or Ki67+ brown nuclei: arrowheads) mostly in p24− cells. Note that most p24+ cells also formed syncytia (multinucleated giant cells) and they are negative for proliferation markers. (**F, G**) BaL Vpr+ infected microglia show BrdU+ nuclei in p24− cells (*arrowheads* in **F**), whereas double-positive cells are readily detected in cultures infected with BaL Vpr− (*arrow* in **G** inset). (*H*,*I*) Similar results are obtained with Ki67 as a proliferation marker in cultures infected with BaL Vpr+ and Vpr−. Arrowheads in **H** indicate Ki67+ nuclei in p24− cells in Vpr+ cultures, whereas arrows in **I** indicate Ki67+ nuclei in p24+ cells in Vpr− cultures.

increased in the HIVE group compared to the HIV− group (Figure 3D).

GM-CSF immunoreactivity is elevated in HIV+ and HIVE brains and correlates with Ki67 counts

GM-CSF expression has been shown to be increased in diseased human brains including those with HIVE (Lee *et al*, 1994; Si *et al*, 2002a). Because GM-CSF is the chief growth factor for brain macrophages *in vitro*, we asked whether a relationship exists between GM-CSF expression and Ki67 labeling in human CNS. Sections immunostained for GM-CSF were graded from 0 to 4 based on the number of positive cells and staining intensity, then were correlated with the Ki67 counts ($n = 16$). GM-CSF immunoreactivity was detectable primarily in reactive astrocytes and a subpopulation of inflammatory cells (not shown) as previously described (Lee *et al*, 1994; Si *et al*, 2002a). GM-CSF was detectable in HIV+ and HIVE cases, but not in HIV− cases (Figure 3E). Furthermore, a positive correlation ($P = .004$, $R^2 = .48$ by Pearson correlation) was found between GM-CSF grades and Ki67 counts after eliminating a single outlier (one HIV+ case, high Ki67 with no GM-CSF: *arrowhead*, Figure 3E), based on the Grubb's Test ($Z = 3.92$ > critical Z 5% = 2.59). No significant correlation was detected *with* the outlier included ($P = .2$, $R^2 = .11$).



Mean ± SEM	HIV-	HIV+	HIVE
VA	0.1 ± 0.0	1.1 ± 0.5	1.6 ± 0.6
Par	0.5 ± 0.3	3.5 ± 1.8	3.6 ± 1.2
Total	0.7 ± 0.3	4.6 ± 2.2	5.2 ± 1.5

Figure 3 Ki67+ labeling of human CNS tissue. Postmortem sections from HIVE and control (HIV− and HIV+) brains were immunostained for Ki67 as described in the Materials and Methods. (**A–C**) Examples of Ki67 labeling of HIV−, HIV+, and HIVE brains are shown. Brown Ki67+ nuclei are rare but are more frequently noted in HIV+ and HIVE brains. Note the increased cellularity in HIVE brain representing a microglial nodule in white matter in **C**. Insets in **B** are high-power views of Ki67+ glial cells (*left*) and endothelial cells (*right*), whereas the inset in **C** shows a Ki67+ astrocyte. The scale bars represent 50 μm in all. (**D**) Ki67+ cell counts were compiled from HIV− ($n = 5$), HIV+ ($n = 8$), and HIVE ($n = 10$) and expressed as median ± range (25% to 75% quartiles shown in box), as well as mean ± SEM per mm^2 white matter area (table below). Vessel-associated and parenchymal cells were analyzed separately. Intravascular cells were excluded. By ANOVA, the differences between HIV− and HIVE for vessel-associated (**$P < .01$) and total Ki67 counts (*$P < .05$) were significant. (**E**) The correlation between Ki67 counts and GM-CSF immunoreactivity. To determine whether there is a relationship between GM-CSF expression and cell proliferation, Pearson correlation analysis was performed on adjacent tissue sections labeled for GM-CSF (grades 0 to 4) and Ki67 (number per mm^2), as described in the Materials and Methods. The analysis showed a strong positive correlation ($P = .004$, $R^2 = .48$) between the two, after an outlier (a HIV+ case, marked by an arrowhead) was excluded.

Identification of Ki67+ macrophages and microglia in HIVE and the relationship to infection

To determine the relationship between cell proliferation and HIV infection in total brain macrophages, we examined Ki67, CD68, and p24 in HIVE cases. First, in order to examine proliferating macrophages, sections were double-labeled for Ki67 and CD68 (total macrophage marker). In another set of sections (from the same tissue block as Ki67/CD68 in all cases except for one), double labeling for Ki67 and p24 was performed to determine the number of Ki67+ infected cells. Single- or double-labeled cells were counted and expressed as the average number per 400× microscope field. The results are summarized in Figure 4. A good correspondence was noted among Ki67+ cell counts when sections from the same blocks were repeatedly stained (Figure 4G). Sections double-labeled for CD68 and p24 showed that p24+ cells were also

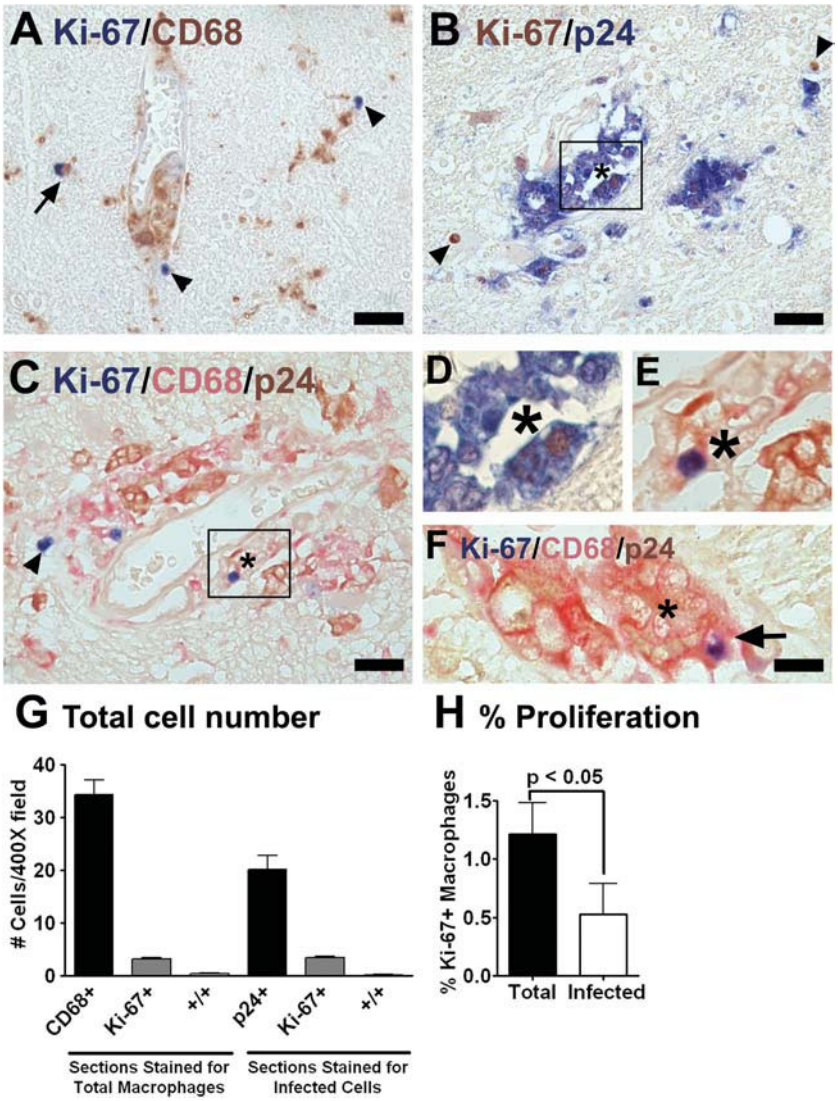


Figure 4 Cell-specific analysis of Ki67+ cells in HIVE. HIVE sections were double- or triple-labeled to determine proliferation among total macrophages (CD68+) and HIV-infected (p24+) cells, as described in the Materials and Methods. Chromogens were switched to confirm reproducibility and specificity of the immunohistochemistry. The figure labels match the color of the chromogen used for each antigen. (**A**) A section double-labeled for Ki67 and CD68 demonstrates Ki67+ nuclei in CD68− (*arrowheads*) and CD68+ (*arrow*) cells. (**B**) An area double-labeled for Ki67 and p24 shows Ki67+ nuclei in p24− (*arrowheads*) and p24+ (*asterisk*) cells. A higher magnification of the same area is shown in (**D**). (**C**) A perivascular cuff is triple-labeled for Ki67, CD68, and p24. All p24+ cells are also CD68+. A single Ki67+ cell (*arrowhead*) and a triple-positive cell (*asterisk*) are shown, with a higher magnification of the latter shown in (**E**). Another triple-labeled cell is shown in (**F**). The vast majority of p24+ cells are Ki67−. The scale bar represents 25 (**A–C**) or 10 (**D–F**) μm. (**G**) Eight HIVE cases were double-labeled for CD68/Ki67 and p24/Ki67 and single- or double-positive cells were counted as described in the Materials and Methods. The percent Ki67+ cells among total (CD68+) and infected (p24+) macrophages were calculated and shown in **H**. They show that the proliferation of the infected cell population is significantly lower than in the total population ($P < .05$).

CD68+ (Figure 4C, E, and F), confirming the identity of the infected cells as macrophages and microglia. The number of CD68+ cells in HIVE cases was high and significant numbers of p24+ cells were also detected in these sections (Figure 4). The average percent of Ki67+ macrophages (Ki67+/CD68+ cell number divided by total CD68+ cell number) in the eight HIVE cases was 1.2% ± 0.3% (Figure 4H). However, the average percent of Ki67+ infected cells (Ki67+/p24+ cell number divided by total p24+ cell number) was only 0.5% ± 0.2%. The difference in Ki67 frequency in the two populations (total versus infected macrophages) was statistically significant ($P < .05$, paired t test).

Analysis of a case with high levels of microglial proliferation

CD68 expression is localized to cytoplasmic organelles, most notably lysosomes (Saito *et al*, 1991). In ramified microglia, CD68 immunoreactivity often appears as a series of discontinuous dots, making the identification of nuclear Ki67+ cells

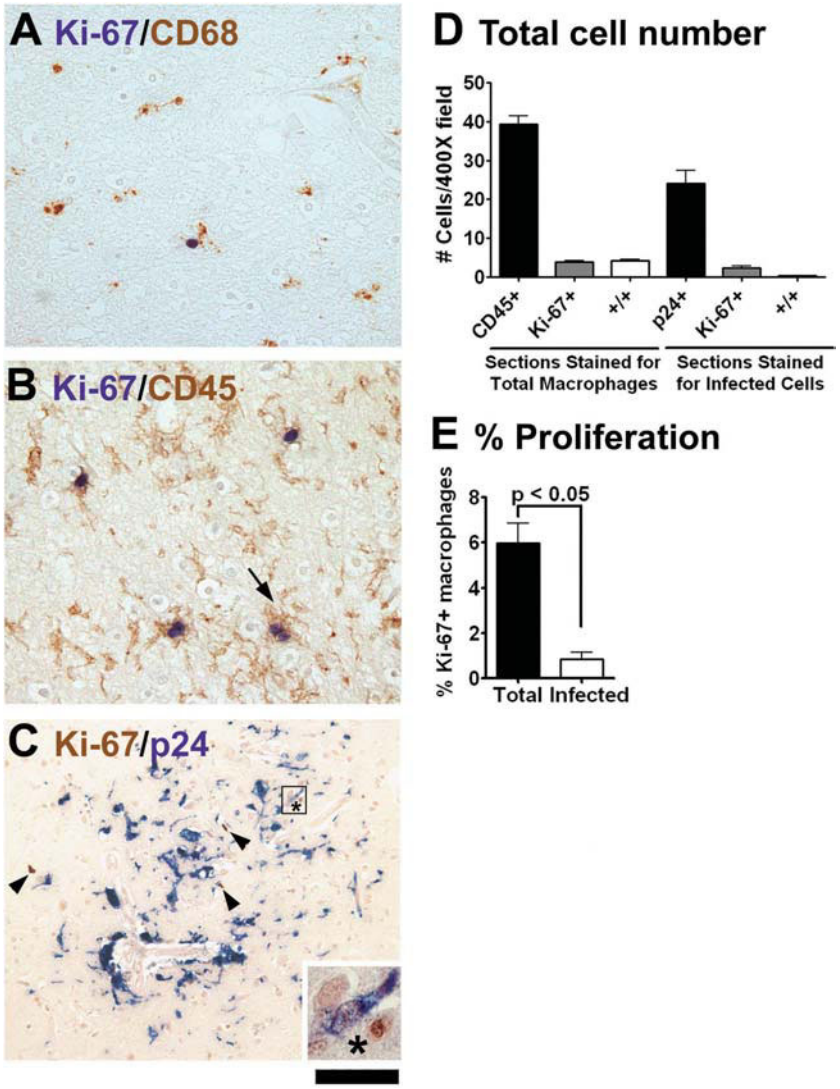


Figure 5 Case analysis of macrophage/microglial proliferation. Multiple regions of a single case with a high microglial proliferation rate were analyzed in detail with double labeling for Ki67/CD45RB or Ki67/CD68. The figure labels indicate the color of the chromogen for each antigen. (**A**) Ki67/CD68 double labeling illustrates a double-positive cell in the center of the field. (**B**) Ki67/CD45 double labeling shows Ki67+ nuclei, all of which belong to highly ramified CD45RB+ microglial cells. One of them is binucleated (*arrow*), suggesting a recently completed nuclear division. A substantial number of Ki67+ microglia were binucleated elsewhere (not shown). (**C**) A microglial nodule in the gray matter double-labeled for Ki67/p24 is shown at a lower magnification. Ki67+ nuclei (*arrowheads*) are among uninfected cells (p24−: *inset*, *asterisk*). The scale bar represents 50 μm in **A** and **B**, 200 μm in **C**, and 20 μm in the inset. (**D**) Cell counting was performed as described in the Materials and Methods and the numbers are expressed as stated in Figure 4 legend. A comparison between CD45RB/Ki67-stained sections and p24/Ki67-stained sections in five different brain regions demonstrates a good correspondence in Ki67 labeling and high levels of productive infection (p24+). The vast majority of Ki67+ cells are also CD45RB+, whereas Ki67+ cells are rarely p24+. (E) The percent Ki67+ cells was calculated among total and infected macrophages and was shown to be significantly decreased in infected macrophages.

as microglia often difficult (for example, see Figure 4A). We surmised that the use of a membrane marker that is highly expressed in resting microglia could eliminate this problem. We therefore adopted CD45RB, which we and others have found to be expressed in microglia in normal brain (Akiyama *et al*, 1994; Cosenza-Nashat *et al*, 2006a). A study of three cases demonstrated that the use of CD45RB indeed increased the number of double-labeled (Ki67/CD45RB) cells as compared to CD68, without significantly increasing the total number of macrophages detected by either marker (data not shown). We thus analyzed a single HIVE case (which also had primary CNS lymphoma) with very high parenchymal Ki67 counts in more detail.

Five brain regions (cerebral cortex and white matter, not including the section of lymphoma) were double-labeled for Ki67/CD45RB and Ki67/p24, and the cells were analyzed as described for Ki67/CD68. As shown in Figure 5A and B, the use of α-CD45RB facilitated the visualization of ramified parenchymal microglia and the identification of Ki67+ cells as

microglia. In a representative section, the average number of double-labeled cells rose from 0.5 (CD68) to 3.6 (CD45) per microscopic field, representing 15% and 88% of total Ki67+ cells, respectively. In sections double-labeled for Ki67/p24, p24+ cells were numerous, whereas Ki67+/p24+ cells were very rare (Figure 5C and D). The percent of Ki67+ cells among CD45RB+ microglia and macrophages for the five brain regions was 6% ± 0.9% (mean ± SEM), whereas the percent Ki67+ cells among p24+ cells was 0.9% ± 0.3% (Figure 5D and E; $P < .05$, paired t test). These data show that even in an environment where macrophage proliferation is abundant, infected cells remain refractory to proliferative stimuli. We noticed a slight increase in the number of total macrophages studied with CD45RB as compared to the number of CD68+ cells, although the difference was not statistically significant. We also compared gray and white matter in this case, without significant differences in the numbers of total macrophages or proliferating cells in the two anatomic regions (not shown).

Discussion

In this study, we show that HIV infection causes a cell cycle block in macrophages and microglia. *In vitro*, HIV-infected microglia showed a lower rate of proliferation than uninfected cells, suggesting that viral protein(s) associated with the incoming virion or newly synthesized in microglia must be responsible for the cytostasis. Our results in microglia point to a possible role for Vpr, the HIV protein most often implicated in cell cycle arrest. The reduction in proliferation was seen with both Ki67 (all phases) and BrdU (S phase) labeling. Although Ki67 is expressed in all phases of cell cycle, its expression in G1 phase may be low (Lopez *et al*, 1991; Schafer, 1998; Scholzen and Gerdes, 2000). Together, these results indicate the microglial proliferation block is not G2, but is most likely a G1/S block.

Our double-label studies of microglia demonstrate a nonoverlapping pattern of the host cell growth (Ki67 or BrdU) and the viral growth (p24), hinting at their mutually exclusive nature. This further suggests that host growth arrest might translate into a gain for HIV. Indeed, data from several systems have demonstrated that HIV production is favored by the induced cell cycle block (Goh *et al*, 1998; Kootstra *et al*, 2000; Roberts *et al*, 1997). The observation that GM-CSF promotes the proliferation of uninfected cells and that HIV-producing cells are nonproliferating also explain why GM-CSF does not increase HIV production (Kandanearatchi *et al*, 2002; Kedzierska *et al*, 1998, 2000; Si *et al*, 2002a). These results demonstrate that macrophage proliferation is not only *un*necessary for HIV infection, but is actively suppressed in infected cells.

In order to examine whether HIV infection causes cell cycle block *in vivo*, we analyzed Ki67 expression in postmortem brain sections. Three groups of brains (HIV−, HIV+ but no HIVE, and HIVE) were examined. The overall proliferation index in the CNS was low. Although a modest increase was noted in both the HIV+ and HIVE groups, large case variations were noted in both. Previously, Morris *et al* documented cell proliferation in the CNS of HIV-infected individuals employing proliferating cell nuclear antigen (PCNA) as a marker of proliferating cells (Morris *et al*, 1994). More recently, others have disputed the value of PCNA as a marker of proliferating cells and have instead adopted Ki67 to measure proliferating cells in the CNS (Anthony *et al*, 2005a; Fischer-Smith *et al*, 2004; Williams *et al*, 2002). One such study reported a marginal (and not significant) increase in total Ki67+ cells in HIVE with large case variations (Fischer-Smith *et al*, 2004). The macrophage populations were not separately analyzed in that study. Another study analyzing the Edinburgh cohort found no proliferating microglial cells, although the identification of Ki67+ cells appears to have been done based on morphology (Anthony *et al*, 2005a). Our study, in part, agrees with the previous studies that proliferating cells in the CNS are rare and variable.

In our analysis, we found a positive correlation between the Ki67 counts and the amount of GM-CSF immunoreactivity among all brain groups (with an outlier), suggesting that the availability of growth factor might in part contribute to the case variability in Ki67 counts (Si *et al*, 2002a). This finding does not exclude the contribution of other factors (such as M-CSF, which acts synergistically with GM-CSF [Lee *et al*, 1994]) or the contribution of nonmacrophage cells to the Ki67+ population, but rather indicates the overall proinflammatory, progrowth factor environment in the CNS of HIV+ individuals. Notably, although we had anticipated higher Ki67 counts in the HIVE than the HIV+ group, we found no such increases. We surmised that HIV has both positive and negative impact on cell proliferation, as we have seen *in vitro*.

In order to determine whether a proliferation block exists in infected macrophages *in vivo*, we analyzed HIVE sections for proliferation and infection by double-label immunohistochemistry. The results demonstrated that proliferating CD68+ cells were indeed detectable in six out of eight cases, with an average of 1.2% of total macrophages (CD68+) being Ki67+. The comparison of the CD68+ and the p24+ populations demonstrated a decrease (0.5%, $P < .05$) in Ki67 labeling in the p24+ population, suggesting a cell cycle block in HIV-infected cells. Although low in number, p24+/Ki67+ cells were still detectible. Although we assumed Ki67+ cells as proliferating based on our *in vitro* data, they could still be growth-arrested cells (such as in G2). It is also possible that HIV proteins responsible for cell cycle block (i.e., Vpr) may not be intact in certain individuals (Subbramanian *et al*, 1998; Wang *et al*, 1996; Yedavalli

et al, 1998). The exact nature of the cell cycle block in HIV-infected human brain remains uncertain.

CD68 is perhaps the most frequently used total CNS macrophage marker (Anthony *et al*, 2005b; Cosenza *et al*, 2004; Fischer-Smith *et al*, 2004; Holness and Simmons, 1993). Our analysis comparing two different markers, CD68 (macrophage-specific) and CD45 (CD45RB, all leukocytes) (Akiyama *et al*, 1994; Cosenza-Nashat *et al*, 2006a; Masliah *et al*, 1991) demonstrate certain advantages in adopting an additional marker. In a case with CNS lymphoma with a very high rate of microglial proliferation, Ki67+ microglial cells were much easier to detect when CD45 (88% of total Ki67+) was used as opposed to CD68 (15% of total Ki67+). This was due to the different subcellular localization of the antigens (i.e., Ki67 nuclear, CD45 plasma membrane, CD68 lysosomes, and p24 diffusely cytosolic) and the fact that CD45 (RB) is a sensitive marker of ramified microglia (Cosenza-Nashat *et al*, 2006a). We agree with others that a panel of markers be used to assess the macrophage/microglial populations in the CNS (Anthony *et al*, 2005a).

The mechanism underlying the HIV-induced cell cycle block remains unclear. HIV is an important modulator of growth factor–induced signal transduction. In macrophages infected with HIV, GM-CSF-induced activation of the Jak2/Stat5 pathway is selectively down-regulated (Warby *et al*, 2003). Stat5 is involved in the G1 progression in hematopoietic cells (Matsumura *et al*, 1999) and thus perturbation of Stat5 by HIV remains a plausible mechanism. In addition, we have recently found that the myeloid-specific Src kinase Hck is activated by GM-CSF and is required for GM-CSF–induced macrophage proliferation (Suh *et al*, 2005). Hck is also activated by HIV Nef and Hck serves as a positive regulator of HIV transcription (Kim *et al*, 2006; Komuro *et al*, 2003). Therefore, HIV might hijack Hck for its own use, making it unavailable for microglia. Hck tyrosine kinase might be the molecular switch that regulates the growth of the virus and microglia.

Information on the impact of HIV on cell proliferation in primary human macrophages is scarce. One study (monocyte-derived macrophages) reported a G2M block and apoptosis without distinction between infected and uninfected populations (Wang and Lewis, 2001). To our knowledge, we are the first to demonstrate an HIV-induced cell cycle block in productively infected microglia, as well as in macrophages in human CNS. These studies underscore the importance of examining the infected cell population apart from the uninfected to understand how HIV alters host cell physiology. Together with previous reports delineating protection of infected macrophages from apoptosis, our results demonstrate that CNS tissue macrophage turnover (generation and death) is further restricted in HIV-infected cells (Briggs *et al*, 2001; Cosenza *et al*, 2004; Cosenza-Nashat *et al*, 2006b). Understanding the HIV and host cell interaction in tissues such as CNS would be important in devising an effective strategy for HIV eradication.

Materials and methods

Human fetal microglial culture

Microglial cultures were generated from mixed human fetal CNS cell cultures as described (Kim *et al*, 2006; Lee *et al*, 1992, 1993; Si *et al*, 2002b). All procedures were approved by the Albert Einstein College of Medicine Institutional Review Board. Briefly, a mixed cell suspension was prepared from the cerebral tissue devoid of meninges by dissociating cells with enzymatic digestion and trituration, followed by filtration through nylon mesh of 230- and 130-μm pore sizes. A single cell suspension was plated at 1–10×10^6 cells per ml in DMEM (Cellgro; with 4.5 g/L glucose, 4 mM l-glutamine, and 25 mM HEPES) supplemented with 5% fetal calf serum, antibiotics, and fungizone (complete medium). Microglial cells that spontaneously detach from the mixed cell monolayer were collected by pooling the medium at 2 to 3 weeks. Monolayers of microglia were prepared in 96-well tissue culture plates at 3×10^4 per 0.1 ml medium. Several hours later, cultures were washed to remove nonadherent cells. Microglial culture were highly pure consisting of >98% CD68+ cells.

HIV strains, microglial infection, and GM-CSF treatment

HIV_{ADA} stocks were obtained from the NIH AIDS Repository and were propagated in human peripheral blood mononuclear cells (PBMCs) as described (Kitai *et al*, 2000). Vpr+/Vpr− HIV congenic strains were generated by transfecting 293 cells with pHXB(BaL) obtained from N. Landau (Salk Institute, San Diego, CA), as described (Connor *et al*, 1995; Cosenza-Nashat *et al*, 2006b; Si *et al*, 2002b). Microglia in 96-well plates in triplicate were exposed to HIV-containing supernatant at ~50 ng/ml of input p24 for 16 to 24 h in complete medium devoid of fungizone. Mock infection consisted of treatment of microglia with matching PBMCs or 293 cell supernatants devoid of virus. To induce microglial proliferation, 10 ng/ml GM-CSF was added as described (Lee *et al*, 1994; Suh *et al*, 2005) at the time of HIV exposure, after washout of the virus (16 to 24 h), and again on day 4 post infection. In cultures harvested on day 10, GM-CSF was added again on day 7 after infection.

Ki67, HIV p24, and BrdU immunocytochemistry

Microglial cultures were fixed at day 7 or 10 post infection with ice-cold methanol and were then permeabilized with 0.1% Triton X-100. Cells were treated with 3% H_2O_2 and 5% normal goat serum sequentially. Anti-Ki67 (1:50, MIB-1, a rabbit IgG from DAKO; or 1:25, rabbit IgG from US Biologicals, Swampscott, MA) were used in conjunction

with avidin-biotin complex (ABC) methods to detect proliferating cells. Antibodies to HIV p24 (IgG1, clone Kal-1, 1:5; DAKO) and CD68 (IgG1, clone KP-1, 1:600; DAKO) were used to detect productively HIV-infected cells and macrophages and microglia respectively, as described (Cosenza *et al*, 2002b; Si *et al*, 2002a). For double immunostaining, antibodies were applied sequentially. Staining was developed first using diaminobenzidine (DAB, brown) then nitro blue tetrazolium (NBT; blue; Sigma) or fast red TR/Naphthol AS-MX (pink; Sigma). To detect S-phase cells, microglia were treated with 50 μM of BrdU (Sigma) for 18 h. After fixation, cells were treated with 2 N HCl for 20 min at 37°C prior to blocking with normal goat serum. Anti-BrdU (IgG2a; US Biologicals) was applied at 1:50 followed by alkaline phosphatase-conjugated anti-IgG2a (Southern Biotechnology, Birmingham, AL) at 1:200.

Cell counting and statistics for in vitro studies
The percentages of HIV infected (p24+) and proliferating (Ki67+ or BrdU+) cells were calculated by counting the total positive and negative cells in the entire well at 400× magnification. This was done by dividing each well into ~10 areas by drawing lines on the undersurface of the well. Results are expressed as mean ± SEM of pooled raw data from three separate microglial cases unless otherwise stated. Treatment groups were compared using one-way analysis of variance (ANOVA) with appropriate post hoc analysis using SigmaStat or GraphPad Prism 4.0 software. Proliferation rates in infected and uninfected cell populations were compared using Student's *t* test.

Detection of proliferating cells in postmortem human brain tissue
Paraffin sections from 25 brains were obtained from the Manhattan HIV Brain Bank. they consisted of HIV− ($n = 5$), HIV+ (no encephalitis; $n = 8$), and HIV encephalitis ($n = 11$). For details of the cases studied, see references (Cosenza *et al*, 2002a, 2002b, 2004; Zhao *et al*, 2001, 2004). The HIVE cases included one with neurosyphilis and another with CNS lymphoma, as described previously. One additional HIVE case (47 year old female) with no other known complicating CNS illness was included in this study. HIVE was confirmed by the presence of multinucleated giant cells and HIV p24+ cells. Control brains had no recognizable pathology and were also negative for HIV p24. Brain areas examined were cerebral white matter, most of which came from the frontal lobe. Paraffin sections were boiled in sodium citrate buffer at 95°C for 20 min for antigen retrieval. Single- and double-label immunohistochemistry for Ki67, CD68, and p24 were proceeded as described above for cultured microglia. In selected cases, triple labeling for Ki67, CD68, and p24 was performed sequentially, in that order, using different chromogens as described above. Sections with single-label immunohistochemistry were counterstained with hematoxylin.

Quantitative analysis and statistics in vivo
The total, parenchymal, or vessel-associated (perivascular and endothelial) Ki67+ cells were counted in the entire white matter area of each section single-labeled for Ki67 and were expressed as the number of Ki67+ cells per mm^2. Cell counts from three patient groups (HIV−, HIV+, and HIVE) were compared using one-way ANOVA with a post hoc test. $P < .05$ was considered significant. Eight HIVE cases were double-labeled for Ki67 and the markers of macrophages (CD68) and infected cells (p24). Ki67/CD68 and Ki67/p24 double-labeled, as well as total CD68+, p24+, and Ki67+, cells were counted and averaged from 12 microscopic fields of white matter and are expressed as the number of cells per 400× field. Differences in Ki67 labeling among total CD68+ cells and p24+ cells were compared using Student's *t* test.

GM-CSF immunohistochemistry and quantification
GM-CSF immunohistochemistry was performed using an antibody to human GM-CSF (polyclonal, 1:50; Genzyme, Cambridge, MA) in conjunction with the ABC method, as previously described (Lee *et al*, 1994; Si *et al*, 2002a). Immunoreactivity was graded based on positive cell number and intensity of staining on a relative scale from 0 to 4. Pearson correlation analysis (Graph Pad Prism 4.0) was used to determine whether a correlation existed between GM-CSF immunoreactivity and Ki67+ counts in the 16 cases (HIV− = 3, HIV+ = 6, HIVE = 7) in which both stains were available.

Analysis of a case with high levels of microglial proliferation
We analyzed a single HIVE case using another microglial marker (CD45RB) in conjunction with Ki67 immunohistochemistry. Five brain regions of cerebral cortex and periventricular white matter that had notable HIVE pathology were selected for study, excluding the region containing CNS lymphoma. Staining for CD45RB was performed first using the ABC method as described (Cosenza-Nashat *et al*, 2006a), followed by Ki67 immunohistochemistry. Staining was completed with both DAB and NBT for both CD45RB and Ki67 with similar results. Sections double-labeled for Ki67 and CD45RB or p24 were counted and expressed as average numbers from 12 400× fields as described above for CD68/Ki67 analysis. Lymphocytes (small, strongly CD45RB+ cells) were excluded from the analysis based on morphology. Lymphocytes in HIVE are also known to be p24− (Cosenza-Nashat *et al*, 2006a; Petito *et al*, 2003). Proliferating cells were counted in this case from both gray and white matter. Differences in Ki67 labeling among CD45RB+ and p24+ cells were compared using Student's *t* test.

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