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Increased In Vivo Activation of Microglia and Astrocytes in the Brains of Mice Transgenic for an Infectious R5 Human Immunodeficiency Virus Type 1 Provirus and for CD4-Specific Expression of Human Cyclin T1 in Response to Stimulation by Lipopolysaccharides[▽]

Jinglin Sun,[1,2] Jian Hua Zheng,[1] Mengliang Zhao,[3] Sunhee Lee,[3] and Harris Goldstein[1,2]*

Departments of Microbiology & Immunology[1], Pediatrics,[2] and Pathology,[3] Albert Einstein College of Medicine, Bronx, New York 10461

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Inflammatory mediators and viral products produced by human immunodeficiency virus (HIV)-infected microglia and astrocytes perturb the function and viability of adjacent uninfected neuronal and glial cells and contribute to the pathogenesis of HIV-associated neurocognitive disorders (HAND). In vivo exposure to lipopolysaccharide (LPS) activates parenchymal microglia and astrocytes and induces cytokine and chemokine production in the brain. HIV-infected individuals display increased circulating LPS levels due to microbial translocation across a compromised mucosa barrier. We hypothesized that HIV-infected microglia and astrocytes display increased sensitivity to the proinflammatory effects of LPS, and this combines with the increased levels of systemic LPS in HIV-infected individuals to contribute to the development of HAND. To examine this possibility, we determined the in vivo responsiveness of HIV-infected microglia and astrocytes to LPS using our mouse model, JR-CSF/human cyclin T1 (JR-CSF/hu-cycT1) mice, which are transgenic for both an integrated full-length infectious HIV type 1 (HIV-1) provirus derived from the primary R5-tropic clinical isolate HIV-1_{JR-CSF} regulated by the endogenous HIV-1 long terminal repeat and the hu-cycT1 gene under the control of a CD4 promoter. In the current report, we demonstrated that in vivo-administered LPS more potently activated JR-CSF/hu-cycT1 mouse microglia and astrocytes and induced a significantly higher degree of monocyte chemoattractant protein production by JR-CSF/hu-cycT1 astrocytes compared to that of the in vivo LPS response of control littermate mouse microglia and astrocytes. These results indicate that HIV infection increases the sensitivity of microglia and astrocytes to inflammatory stimulation and support the use of these mice as a model to investigate various aspects of the in vivo mechanism of HIV-induced neuronal dysfunction.

Human immunodeficiency virus (HIV) infection causes a broad range of HIV-associated neurocognitive disorders, including asymptomatic neurocognitive impairment, HIV-associated mild neurocognitive disorder, and HIV-associated dementia (HAD) (2). HAD is a devastating neurological disease that is a frequent consequence of HIV infection (46, 49). This clinical syndrome is associated with the pathological findings of HIV type 1 (HIV-1) encephalitis (HIVE), specifically multinucleated giant cell formation, microglial nodules, astrogliosis, myelin pallor, and neocortical atrophy (54). HIV-1 enters the central nervous system (CNS) soon after the initiation of HIV-1 infection, predominantly by the transmigration of HIV-1-infected cells from the systemic circulation across the blood-brain barrier (BBB) (18, 24, 36, 50). Although HIV enters into the brain early in the course of infection, CNS disease does not develop in HIV-infected individuals until many years later, usually in association with the depletion of $CD4^+$ T cells and the resultant development of immunodeficiency (54). Despite the dramatic reduction in the incidence of neurological manifestations of HIV-1 infection due to the potent antiviral effects of highly active antiretroviral therapy (HAART), some patients treated with HAART develop a variant of HIV leukoencephalopathy that is characterized by the intense perivascular infiltration of HIV-infected macrophages, very high levels of HIV in the brain, and extensive white-matter destruction (39). The development of HIV CNS disease despite HAART may be a consequence of several factors, including prolonged patient survival; continued HIV replication due to the poor penetration of antiretroviral agents into the brain; the emergence of drug-resistant isolates; the possible toxic effects of HAART on cerebrovascular endothelium, astroglial cells, and the white matter (39); and/or immune reconstitution inflammatory syndrome (73).

A major factor contributing to the development of HAD is the chronic activation of macrophages and astrocytes resident in brain microglia that is associated with the production of cytotoxic factors, including proinflammatory cytokines and reactive oxygen intermediates (33). In vivo exposure to LPS induces microglia activation and cytokine and chemokine production in the brain (81). We postulated that microglial and astrocyte activation is induced by the markedly increased plasma LPS levels detected in HIV-infected individuals due to microbial translocation across a mucosa barrier compromised by the HIV-induced depletion of mucosal CD4 T lymphocytes (8, 17). The mean plasma LPS level of 75 pg/ml measured in HIV progressors is markedly higher than the level (14 pg/ml) that induced systemic immune activation and elevated levels of

* Corresponding author. Mailing address: Albert Einstein College of Medicine, Forchheimer Building, Room 408, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-2156. Fax: (718) 430-2374. E-mail: hgoldste@aecom.yu.edu.

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inflammatory cytokines in volunteers intravenously injected with endotoxin (17). This increased systemic LPS in the HIV progressors was associated with the in vivo activation of their innate and adaptive immune systems (8, 17). We hypothesized that the increased levels of systemic LPS in HIV-infected individuals permit the passage of LPS across the BBB into the brain and play a contributory role in the development of HAD that is further exacerbated by the increased sensitivity of HIV-infected microglia and astrocytes to the proinflammatory effects of LPS.

The investigation of the role of microglia and astrocytes in HIV-mediated neurotoxicity is complicated by differences between the in vivo behavior of CNS inflammatory cells and their in vitro behavior in tissue culture (62). To develop an in vivo model for studying the pathogenesis of HIV infection, we circumvented the entry block of HIV into mouse cells by developing transgenic mice that carry an integrated full-length infectious HIV-1 provirus derived from the primary R5-tropic clinical isolate HIV-$1_{JR\text{-}CSF}$ that is under the control of the endogenous HIV-1 long terminal repeat (LTR) (JR-CSF mice); JR-CSF mice are populated with T lymphocytes and monocytes that produce infectious HIV-1 and display plasma viremia of up to 10^5 HIV copies/ml (10, 51). The LTR-regulated HIV-1 transgene is expressed in the brains and microglia of JR-CSF mice and is responsive to in vivo and in vitro LPS stimulation, and its expression is associated with increased in vivo and in vitro CCL2/monocyte chemoattractant protein (MCP-1) gene expression in response to LPS (75). HIV production in this HIV transgenic model was increased by the coexpression of a transgene encoding hu-cycT1 under the control of a CD4 promoter. The expression of the hu-cycT1 transgene circumvents the inability of mouse cells to support the function of Tat, the transactivator of HIV replication, due to the structural differences between mouse and hu-cycT1 that prevent mouse cyclin T1 from recruiting Tat to the transcription initiation site (7, 22). CD4 T lymphocytes and myeloid lineage cells from these double-transgenic JR-CSF/hu-cycT1 mice displayed markedly increased HIV production compared to that of the JR-CSF mice (69). We postulated that the JR-CSF/hu-cycT1 mice can be used to study the in vivo effect of HIV expression and the contribution of Tat-mediated transcriptional activation on LPS-mediated activation of microglia and astrocytes. In the current report, we demonstrated that microglia and astrocytes in the brains of JR-CSF/hu-cycT1 mice displayed increased in vivo activation and MCP-1 production in response to LPS. These results indicated that HIV infection increases the sensitivity of microglia and astrocytes to LPS stimulation and support the possible role of the increased systemic LPS detected in HIV-infected individuals as a contributory factor for the development of HIV-induced neuronal dysfunction.

MATERIALS AND METHODS

Construction of mice transgenic for hu-cycT1 and HIV-$1_{JR\text{-}CSF}$. The hu-cycT1 mice were constructed and generated as described previously (69). After the 1.15-kb cDNA fragment encoding hu-cycT1 was cloned into an engineered SalI site in a murine CD4 expression cassette targeting expression to CD4 T lymphocytes, macrophages, and dendritic cells (34), the plasmid was linearized and microinjected into the pronuclei of fertilized embryos derived from FVB × C57/B6 mouse crosses. Transgenic founders were identified by the PCR analysis of genomic DNA extracted from tails using a primer pair specific for the amplification of hu-cycT1 DNA; the specific expression of the hu-cycT1 gene by their $CD4^+$ T cells and monocytes was detected by Western blotting as described previously (69). The JR-CSF mice were constructed and characterized as previously described (10) and were crossed with the hu-cycT1 mice to obtain JR-CSF/hu-cycT1 mice that were transgenic for hu-cycT1 controlled by the CD4 promoter and the HIV-$1_{JR\text{-}CSF}$ provirus regulated by the endogenous HIV-1 LTR.

In vivo treatment with LPS by intracranial and i.p. injection. After 2- to 4-month-old transgenic mice or control littermates were anesthetized with pentobarbital (40 to 80 mg/kg of body weight), 5 μg of LPS (from *Escherichia coli* 0111:B4; Sigma, St. Louis, MO) or phosphate-buffered saline (PBS) was injected in a volume of 10 μl into their right frontal cortexes. The mice were killed 3 days later, and their brains were harvested. Another group of mice was injected intraperitoneally (i.p.) with LPS (3 mg/kg) in a volume of 100 μl and sacrificed 5 days later, and their brains were collected. The brains were examined microscopically after immunochemical or immunofluorescent staining.

Real-time PCR assay for quantification of HIV RNA. The real-time PCR assay was performed using a TaqMan one-step RT-PCR protocol and RT-PCR Master Mix reagent kit (Applied Biosystems). After an initial reverse transcription reaction at 48°C for 30 min and at 95°C for 10 min, PCR amplification was performed for 40 cycles (with each cycle consisting of 95°C for 15 s and 60°C for 1 min) in the presence of a primer-probe set specific for HIV-1 quantification that was designed to amplify and hybridize to conserved regions of the *gag* gene. The primer pair used was 6F (5′-CATGTTTTCAGCATTATCAGAAGGA-3′) and 84R (5′-TGCTTGATGTCCCCCCACT-3′) (600 nM), and the probe used was 5′FAM-CCACCCCACAAGATTTAAACACCATGCTAA-Q 3′ (100 nM); FAM is the reporter 6-carboxyfluorescein group, and Q represents a 6-carboxytetramethylrhodamine group quencher conjugated through a linker arm nucleotide. HIV-1 RNA standards were generated using the RiboMax Express large-scale RNA production system (Promega, Madison, WI) from a Bluescript II/KS+/− plasmid that contained a T7 promoter-regulated 1,820-bp fragment of HIV-1 PYK–JR-CSF *gag* (NarI to EcoRV) and were purified using an RNeasy kit (Qiagen). The HIV RNA transcripts were quantified by measuring the specific absorbance at 260 nm, diluted to 10^6 copies/μl, divided into aliquots, and stored at −80°C. The aliquots of HIV-1 RNA transcripts were diluted to generate standards ranging from 10^6 to 0.3 copies/10 μl to produce a standard curve for each single-copy assay as described previously (58, 59). Threshold cycle values were plotted as a function of the input transcript copy number, and a standard curve was generated by linear regression using ABI 7900 sequence detection software (Applied Biosystems) (52).

Detection of HIV p24 antigen in mouse brains by immunofluorescence. After mice were perfused with PBS, their brains were harvested, embedded in Tissue-Tek OCT compound, frozen, and cut by a cryostat into 10-μm-thick sections. The sections were fixed for 20 min at −20°C in ethanol and permeabilized by incubation in PBS containing 0.2% Triton X-100 (PBT) for 20 min at room temperature. Nonspecific binding sites were blocked with PBT containing 0.2% bovine serum albumin (BSA) and 5% heat-inactivated normal goat serum for 30 min at room temperature. Unlabeled mouse anti-p24 immunoglobulin G (IgG) was incubated with the Zenon labeling reagent (Invitrogen, Carlsbad, CA), which contained an Alexa Fluor 647-labeled Fab fragment directed against the Fc portion of mouse IgG. After the labeled Fab fragment bound to the Fc portion of the anti-p24 IgG, the excess labeled Fab fragment was complexed and neutralized by incubation with an excess of nonspecific IgG. The Zenon labeling complex and antibody to isolectin B4 (Sigma), a microglia/macrophage cell marker, were diluted in PBT to the desired working concentration, incubated with the sections for 1 to 2 h at room temperature, and washed for 5 min in PBS at room temperature. The tissue sections were fixed in 4% formaldehyde solution in PBS for 15 min at room temperature, counterstained, and mounted with 4′,6′-diamidino-2-phenylindole (DAPI)-containing ProLong Gold antifade reagent (Molecular Probes). The sections were analyzed with a Leica AOBS laser scanning confocal microscope system, and photomicrographs of representative fields were taken.

Immunohistological analysis of mouse brains for expression of Iba-1 and GFAP. Following the perfusion of the mice by intracardiac injection of PBS, their brains were harvested, fixed in 10% buffered formalin, bisected coronally, and embedded in paraffin. Five-micrometer sections were cut, and the slides were deparaffinized in xylene and then dehydrated by incubation in graded ethanol solutions. Slides were boiled in sodium citrate buffer for 20 min for antigen retrieval and then incubated sequentially with 3% H_2O_2 in PBS for 30 min and 10% normal goat serum for 1 h. The primary antibodies used were rabbit anti-mouse Iba-1 (Wako Chemical USA, Richmond, VA) and rabbit anti-mouse GFAP (BioGenex Laboratories, San Ramon, CA). The slides were incubated with the indicated antibody overnight at 4°C, followed by sequential incubation with the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit

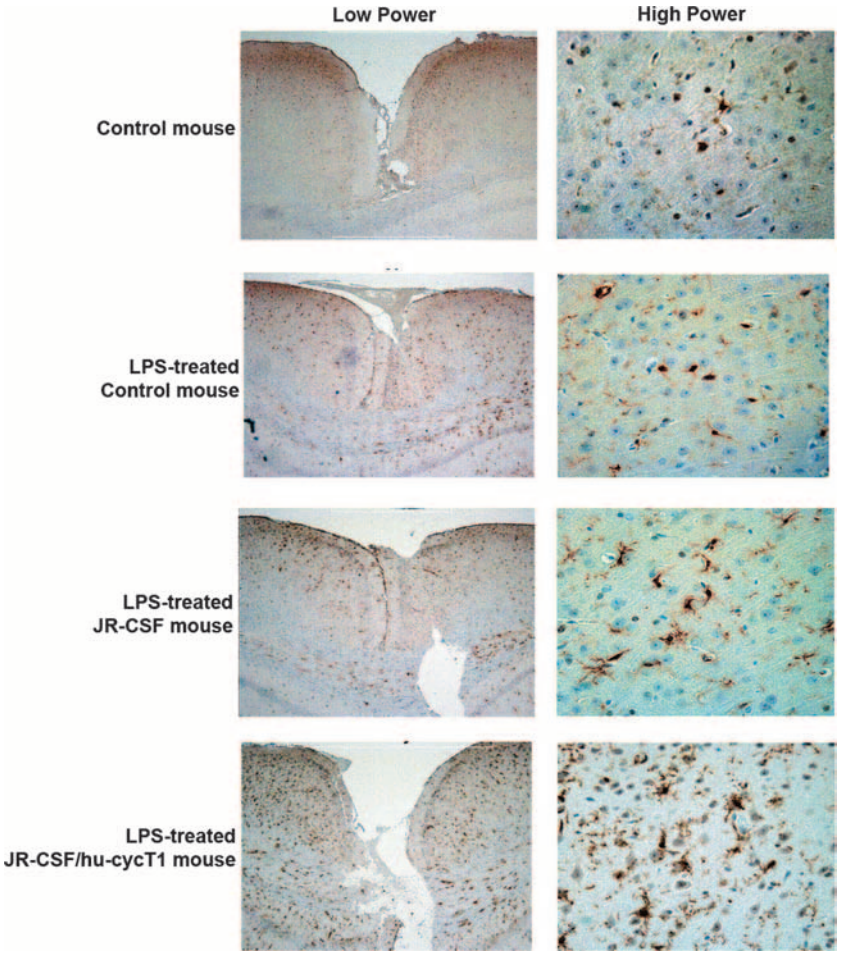

FIG. 1. Increased activation of JR-CSF/hu-cycT1 mouse microglia in response to LPS. JR-CSF mice, JR-CSF/hu-cycT1 mice, or control littermates ($n = 3$ mice/group) were injected intracranially with LPS. The mice were killed 3 days later, and paraffin sections from their brains were stained by immunocytochemistry for the expression of the microglia/macrophage activation marker Iba-1. Photomicrographs of low-power views (left) and high-power views (right) of representative fields of an uninjected control mouse brain and LPS-injected control, JR-CSF, and JR-CSF/hu-cycT1 mouse brains are shown.

IgG, and the chromogen substrate diaminobenzidine (Vector Laboratories, Burlingame, CA), and then the slides were counterstained with hematoxylin.

Detection of mouse brain CCL2/MCP-1 production by mouse astrocytes. After mice were perfused by an intracardiac injection of PBS, their brains were harvested, fixed in 10% buffered formalin, bisected coronally, and embedded in paraffin. Five-micrometer sections were deparaffinized in xylene, dehydrated by incubation in graded ethanol solutions, and treated with pepsin (Zymed) at 37°C for 10 min. After nonspecific reactivity was blocked by incubation with sheep serum and BSA blocking solution, the slides were incubated with rabbit anti-mouse GFAP (Sigma) and goat anti-mouse CCL2/MCP-1 (Santa Cruz Biotechnology) at optimized concentrations, washed, incubated with Cy3-conjugated sheep anti-rabbit IgG (Sigma) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology), washed again, and mounted with DAPI-containing ProLong Gold antifade reagent (Molecular Probes). The sections were analyzed with a Leica AOBS laser scanning confocal microscope system, and photomicrographs of representative fields were taken.

Statistical analysis of the data. The Student's *t* test for unpaired data was used for all statistical comparisons made. Significance was assigned for $P < 0.05$.

RESULTS

Increased responsiveness of JR-CSF/hu-CycT1 mouse microglia and astrocytes to in vivo LPS stimulation. The increased activation and proliferation of brain parenchymal cells, including microglia and astrocytes, are a prominent feature of HAD. We hypothesized that the increased levels of systemic LPS present in HIV-infected individuals that activate the innate and adaptive immune systems (8) also cross the BBB and activate HIV-infected microglia and astrocytes in the brain. One approach used to induce an inflammatory state in the mouse brain is to inject LPS directly into the brain (29, 30). We applied this approach using our novel HIV transgenic mouse models to investigate the effects of HIV expression and the contribution of the Tat-mediated transactivation of the HIV provirus on the in vivo capacity of LPS to activate microglia and astrocytes (Fig. 1). In the absence of LPS stimulation, the microglia in the mouse brains displayed the normal appearance of lightly stained, resting, ramified microglia with thin delicate processes. An intracranial injection of LPS induced the in vivo activation of microglia, as indicated by the appearance of rounded, amoeboid microglia that stained intensely with Iba-1, a protein upregulated in activated microglia (31). There was a clear hierarchy in the degree of microglial activation in response to LPS, as manifested by the number and size

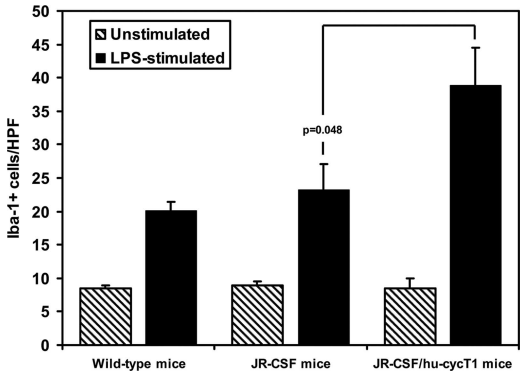

FIG. 2. Quantitative analysis of microglial activation induced by LPS. Iba-1-positive cells were counted in the perihippocampal cortex region in three ×400-magnification fields per brain section and are expressed as the average numbers (± standard errors of the means) of Iba-1-positive cells per HPF of three sections/mouse brain/group (n = 3 mice/group). The fields evaluated were located in similar regions of the cortex for each section.

of Iba-1-positive microglia in the mouse brains. The greatest degree of microglia activation was observed in JR-CSF/hu-cycT1 mouse brains, followed by JR-CSF mouse brains and wild-type littermate brains. Microglia of JR-CSF/hu-cycT1 mice displayed a more intense Iba-1 staining than did microglia from JR-CSF mice or control littermates, and this staining was associated with a hyperramified and bushy appearance with short thick processes (Fig. 1). The degree of microglial activation in the mouse brain was quantified by enumerating Iba-1-positive cells in multiple ×400-magnification high-power fields (HPF) of the brains of unstimulated and LPS-stimulated JR-CSF mice (n = 3 mice/group), JR-CSF/hu-cycT1 mice (n = 3 mice/group), and control wild-type littermates (n = 3 mice/group). As shown in Fig. 2, while LPS stimulation significantly increased the number of Iba-1-positive cells in the brains of wild-type mice (P = 0.001), JR-CSF mice (P = 0.02), and JR-CSF/hu-cycT1 mice (P = 0.004), the number of Iba-1-positive cells detected in the brains of JR-CSF/hu-cycT1 mice was almost twofold higher than the number of Iba-1-positive cells observed in the brains of wild-type mice (P = 0.02) and JR-CSF mice (P = 0.048). This indicated that JR-CSF/hu-cycT1 mouse microglia were more responsive to LPS than wild-type and JR-CSF mouse microglia.

We next examined the impact of the expression of the HIV transgene, with and without the support of Tat-mediated transactivation, on the in vivo activation of astrocytes in response to LPS by evaluating the astrocyte expression of GFAP, an astrocyte-specific activation marker. In the brains of untreated mice, GFAP immunoreactivity was observed in the white matter, and only minimal GFAP immunoreactivity was detected in cortical gray matter (Fig. 3). LPS treatment increased the intensity of GFAP immunoreactivity in the cortical gray matter of all LPS-treated mice (Fig. 3). However, the most intense GFAP staining associated with the formation of a glial network across all layers of the cerebral cortex was observed in the brains of the LPS-treated JR-CSF/hu-cycT1 mice (Fig. 3). As shown in Fig. 4, the LPS-induced activation of astrocytes was quantified by enumerating GFAP-positive cells in several sections of brain from groups of wild-type, JR-CSF, and JR-CSF/hu-cycT1 mice

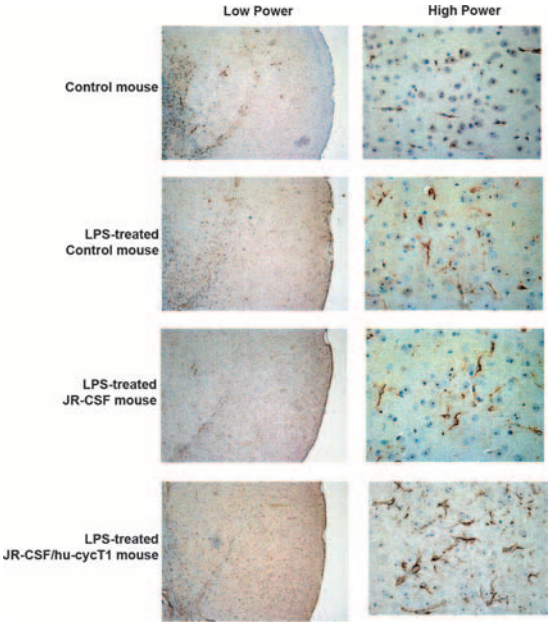

FIG. 3. Increased in vivo activation of JR-CSF/hu-cycT1 astrocytes in response to LPS. JR-CSF/hu-cycT1 mice, JR-CSF mice, or control littermates (n = 3 mice/group) were injected intracranially with LPS. The mice were killed 3 days later, and paraffin-embedded sections of the brain were stained by immunocytochemistry for the astrocyte-specific marker GFAP. Photomicrographs of low-power views (left) and high-power views (right) of representative fields of an uninjected control mouse brain and LPS-injected control, JR-CSF, and JR-CSF/hu-cycT1 mouse brains are shown.

(3 mice/group). Treatment with LPS significantly increased the number of GFAP-positive cells by fourfold in the brains of wild-type mice (P = 0.007) and JR-CSF mice (P = 0.001) and by sixfold in the brains of JR-CSF/hu-cycT1 mice (P = 0.001). The number of GFAP-positive cells was significantly increased by 1.5-fold in LPS-stimulated JR-CSF/hu-cycT1 mice compared to that of wild-type mice (P = 0.05) and JR-CSF mice

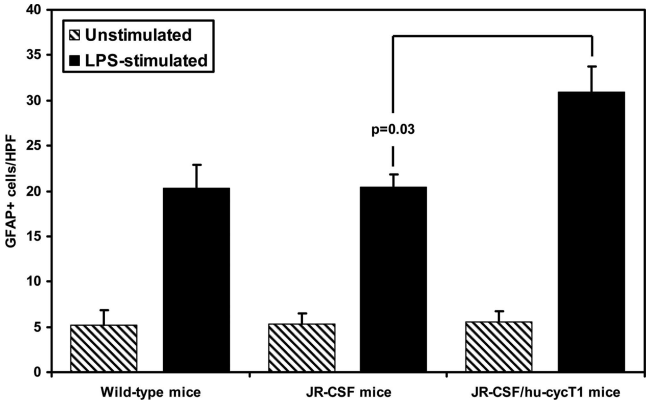

FIG. 4. Quantitative analysis of in vivo astrocyte activation induced by LPS. GFAP-positive cells were counted in the perihippocampal cortex region in three ×400-magnification fields per brain section and are expressed as the average numbers (± standard errors of the means) of GFAP-positive cells per HPF of three sections/mouse brain/group (n = 3 mice/group). The fields evaluated were located in similar regions of the cortex for each section.

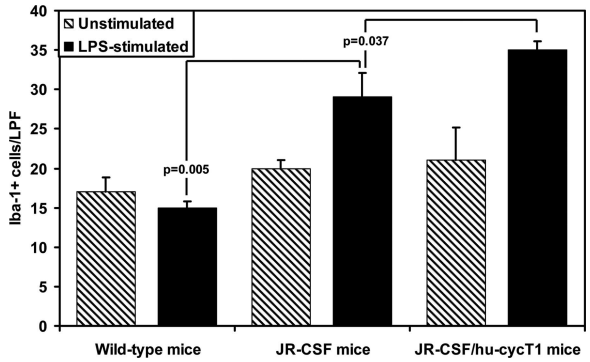

FIG. 5. Increased activation of JR-CSF/hu-cycT1 mouse microglia after i.p. injection of LPS. JR-CSF/hu-cycT1 mice, JR-CSF mice, or control littermates ($n = 3$ mice/group) were injected i.p. with LPS. The mice were killed 5 days later, and paraffin sections from their brains were stained by immunocytochemistry for the expression of the microglia/macrophage activation marker Iba-1. Iba-1-positive cells were counted in three ×100-magnification fields in the cortex area around the hippocampus and are expressed as the average numbers (± standard errors of the means) of Iba-1-positive cells per low-power field (LPF) of three sections/mouse brains of each group. The fields evaluated were located in similar regions of the cortex for each section.

($P = 0.03$), indicating that JR-CSF/hu-cycT1 astrocytes were more responsive to LPS than wild-type or JR-CSF mouse astrocytes.

To evaluate the effect of systemic LPS on microglial and astrocyte activation, we investigated whether similar responses were observed when LPS was administered to the mice by peripheral injection. Two-month-old transgenic mice or control littermates were injected i.p. with LPS, and 5 days later the mice were killed and their brains were examined histologically. Microglial activation induced by the LPS was quantified by enumerating Iba-1-positive cells in multiple fields of the brains of unstimulated and LPS-stimulated JR-CSF mice, JR-CSF/hu-cycT1 mice, and control wild-type littermates (Fig. 5). Not surprisingly, a single i.p. dose of LPS did not induce as potent an inflammatory stimulus for activating microglia as did a single intracranial dose of LPS. After the i.p. LPS injection, the number of Iba-1-positive cells in the brains of wild-type mice was equivalent to the numbers in uninjected mice ($P = 0.38$). The number of Iba-1-positive cells in the brains of JR-CSF mice was almost twofold greater than the number detected in the brains of wild-type mice injected with LPS ($P = 0.005$). The greatest response to i.p. LPS stimulation was observed in JR-CSF/hu-cycT1 mouse brains, which displayed a 2.3- and 1.2-fold increase in the numbers of Iba-1-positive cells compared to that of wild-type mouse brains ($P < 0.0001$) and JR-CSF mouse brains ($P = 0.037$), respectively. No activation of astrocytes as indicated by an increased number of GFAP-staining cells was observed in the brains of any of the mice injected i.p. with LPS (data not shown), indicating that astrocytes are less sensitive to LPS activation than microglia. It is likely that repeated i.p. injections of LPS as opposed to the single dose used in this experiment would better recapitulate the chronic LPS exposure occurring in HIV-infected individuals and would be a more potent stimulator of microglial and astrocyte activation that would be comparable to that observed after a single intracranial injection of LPS.

In vivo LPS treatment induces CCL2/MCP-1 production by JR-CSF/hu-cycT1 mouse astrocytes. We previously demonstrated that treatment with LPS induced the production of significantly higher levels of CCL2/MCP-1 by JR-CSF/hu-cycT1 mouse monocytes compared to those of JR-CSF mouse monocytes (69). We therefore extended those findings to determine if increased in vivo LPS-induced CCL2/MCP-1 production was also observed in the brains of JR-CSF/hu-cycT1 mice as well as to identify the cells in the brain that produced CCL2/MCP-1. Confocal microscopic analysis of the mouse brains stained with DAPI, anti-CCL2/MCP-1, and anti-GFAP

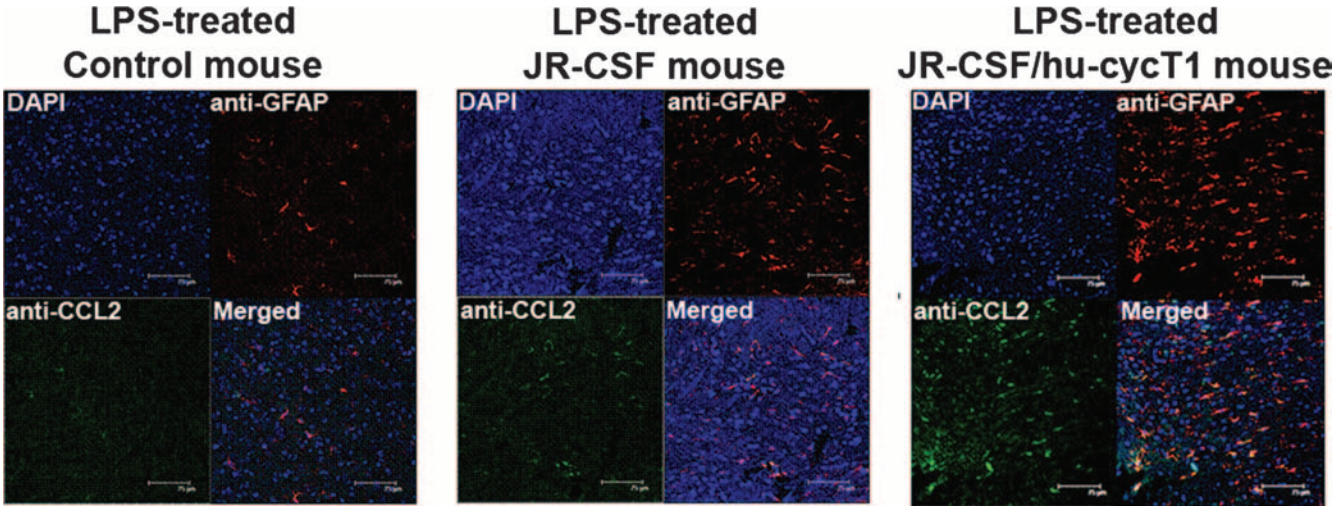

FIG. 6. Microglia from JR-CSF/hu-cycT1 mice produce increased CCL2/MCP-1 in response to LPS. JR-CSF/hu-cycT1 mice, JR-CSF mice, or control littermates ($n = 3$ mice/group) were injected intracranially with LPS. The mice were killed 3 days later, and paraffin sections from the brains were examined by confocal immunofluorescence microscopy after being stained with DAPI (upper left), anti-CCL2/MCP-1 antibody (lower left), and anti-GFAP antibody (upper right). A merged view also is shown (lower right). Photomicrographs (scale bar, 75 μm) of representative fields of LPS-injected control, JR-CSF, and JR-CSF/hu-cycT1 mouse brains are shown.

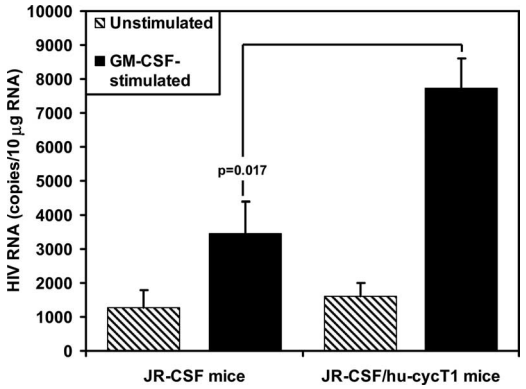

FIG. 7. Systemic GM-CSF increases HIV-1 production in the brains of JR-CSF/hu-cycT1 mice. JR-CSF mice and JR-CSF/hu-cycT1 mice (n = 4 mice/group) were implanted with control B16-F10 cells (unstimulated) or B16-F10–GM-CSF cells (GM-CSF stimulated). Two weeks after implantation, the mouse brains were harvested, and RNA was extracted from the brain tissue and evaluated by real-time PCR to measure the level of HIV-1 production. The data are presented as the average numbers of HIV RNA copies/10 μg brain RNA/group ± standard errors of the means.

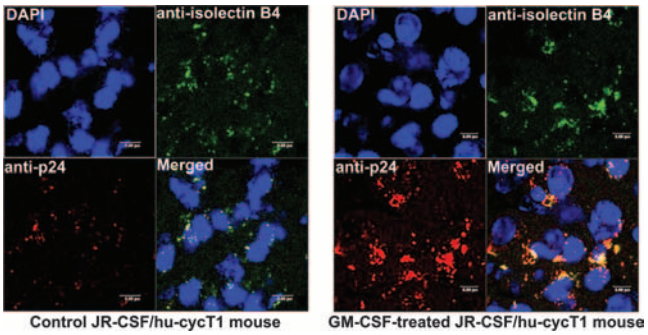

FIG. 8. Systemic GM-CSF increases in vivo HIV-1 p24 antigen production by JR-CSF/hu-cycT1 mouse microglia. JR-CSF/hu-cycT1 mice were untreated (A) or implanted with B16-F10–GM-CSF cells (B). Two weeks later, their brains were examined by confocal immunofluorescence microscopy with DAPI (upper left), FITC-labeled isolectin B4 (upper right), and Alexa Fluor 647-labeled anti-p24 antigen (lower left). A merged view is shown (lower right). Pictures shown are representative of three independent experiments. Photomicrographs (scale bar, 8 μm) of representative fields of unstimulated and GM-CSF-stimulated JR-CSF/hu-cycT1 mouse brains are shown.

and a merged view of anti-CCL2/MCP-1 and anti-GFAP staining of LPS-treated wild-type, JR-CSF, and JR-CSF/hu-CycT1 mice are shown in Fig. 6. Increased numbers of CCL2/MCP-1-stained cells were detected in LPS-stimulated JR-CSF/hu-CycT1 mouse brains compared to that of LPS-stimulated JR-CSF mouse and control wild-type mouse brains, with the majority of the induced CCL2/MCP-1 staining localized to GFAP-positive astrocytes. Thus, the production of CCL2/MCP-1 in response to LPS is increased in astrocytes carrying an integrated Tat-responsive HIV provirus.

JR-CSF/hu-cycT1 mouse microglia are more responsive to in vivo GM-CSF treatment. We previously demonstrated that the CD4 promoter-regulated expression of hu-cycT1 was associated with a marked increase in granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced HIV-1 production by JR-CSF mouse monocytes (69). Inflammatory factors such as LPS induce activated astrocytes to produce GM-CSF, and increased GM-CSF production may play a contributory role in the development of HAD (67). We investigated if the support of Tat responsiveness by the expression of hu-cycT1 also increased HIV production induced by GM-CSF in mouse brains carrying an integrated HIV provirus. Because systemic GM-CSF crosses the BBB into the brain parenchyma (48), we could treat mouse brains continuously with GM-CSF by subcutaneously implanting the mice with a melanoma cell line stably transfected with a GM-CSF expression vector, B16-F10–GM-CSF, which forms a subcutaneous tumor that secretes GM-CSF into the bloodstream and provides steady-state serum levels of GM-CSF of about 100 pg/ml (69). To document that this approach generates a sufficient serum concentration of GM-CSF to cross the BBB into the brain parenchyma, we measured GM-CSF levels in the brains of mice 2 weeks after they were implanted with either B16-F10–GM-CSF cells or control B16-F10 cells. GM-CSF was not detected in the brain lysate of mice implanted with the untransduced B16-F10 cell line. In contrast, equivalently high levels of GM-CSF (P = 0.8) were detected in the brain lysates of JR-CSF mice (mean = 24 ± 1 pg/ml) and JR-CSF/hu-cycT1 mice (mean = 23 ± 1 pg/ml) implanted with the B16-F10–GM-CSF cells. To determine if GM-CSF increased HIV-1 production in the transgenic mouse brains, the level of HIV-1 in the brains of GM-CSF-treated mice was determined by quantifying the number of HIV RNA copies using a real-time PCR assay (Fig. 7). In vivo treatment with GM-CSF significantly increased HIV production by almost threefold in the brains of JR-CSF mice (P = 0.087) and by greater than fivefold in the brains of JR-CSF/hu-cycT1 mice (P = 0.00008). The level of HIV production induced by GM-CSF in the brains of JR-CSF/hu-cycT1 mice was almost twofold higher than the levels induced by GM-CSF in the brains of JR-CSF littermates (P = 0.017).

To identify the cells in the mouse brains that increased the production of HIV in response to GM-CSF, we used confocal microscopy to analyze brain sections double-stained with antibodies to HIV-1 p24 antigen and microglia/macrophage markers (Fig. 8). HIV p24 antigen staining was increased in the brains of B16-F10–GM-CSF-implanted JR-CSF/hu-cycT1 mice compared to that of the brains of untreated JR-CSF/hu-cycT1 mice, particularly in cells also expressing the microglia/macrophage marker isolectin B4. Taken together, these results indicated that GM-CSF present in the systemic circulation crosses the BBB into the brain and activates HIV production by microglia and macrophages. In vivo GM-CSF treatment associated with the support of Tat function was associated with increased in vivo activation of microglia, as indicated by the increased number of Iba-1-positive cells in the brains of GM-CSF-treated JR-CSF/hu-cycT1 mice compared to that of GM-CSF-treated JR-CSF mouse and wild-type control littermate brains (Fig. 9). Microglia activation induced by GM-CSF was quantified by enumerating Iba-1-positive cells in the brains of the treated mice (Fig. 10). While systemic GM-CSF treatment had no significant effect on the number of Iba-1-positive cells in the brains of wild-type or JR-CSF mouse brains, GM-CSF treatment significantly increased (P = 0.015) the number of Iba-1-positive cells in the brains of JR-CSF/hu-cycT1 mice by greater than twofold. These results indicated that increased

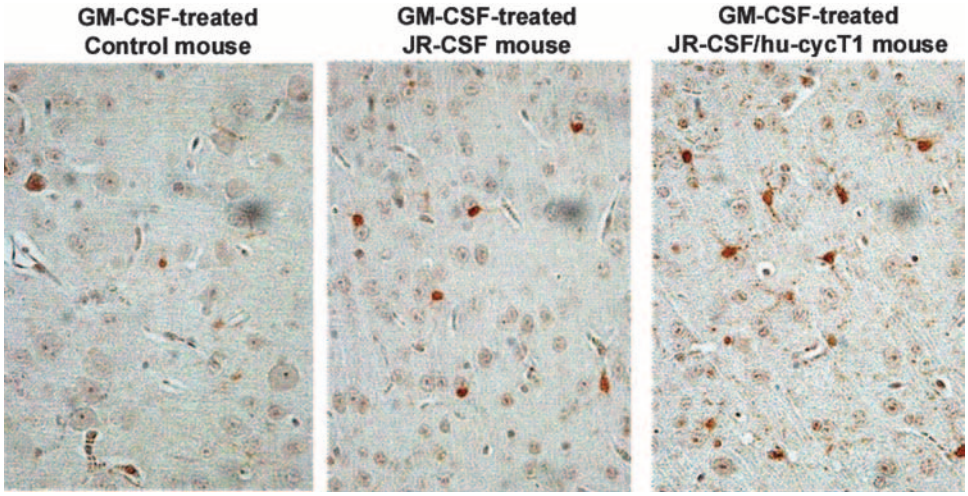

FIG. 9. Microglia from JR-CSF/hu-cycT1 mouse brains display increased activation after in vivo treatment with GM-CSF. JR-CSF mice, JR-CSF/hu-cycT1 mice, or control littermates were implanted with B16-F10–GM-CSF cells. The mice were killed 2 weeks later, and the paraffin sections of the brain were stained by immunocytochemistry for the expression of the microglia activation marker Iba-1. Photomicrographs of representative HPFs of GM-CSF-stimulated control littermate, JR-CSF, and JR-CSF/hu-cycT1 mouse brains are shown.

HIV replication due to the support of Tat function conferred by the expression of hu-cycT1 was associated with the increased activation of microglia in response to GM-CSF. We did not detect increased GFAP staining in the brains of the GM-CSF-treated mice (data not shown), indicating that systemic GM-CSF did not activate astrocytes.

DISCUSSION

Although animal models do not completely recapitulate all aspects of HIV infection in humans, studies using various animal model systems, including simian immunodeficiency virus-infected macaques and pigtail monkeys, feline immunodeficiency virus-infected cats, HIV-infected hu-SCID mouse models, and transgenic mouse models have provided insights into the pathophysiology of HIV-1-mediated neuronal dysfunction and permitted the evaluation of therapies directed toward improving mental function in HIV-infected subjects with cognitive and motor dysfunction (42, 57, 61, 68, 70). Mice, with their well-characterized immune systems, ease of genetic manipulations, and low cost, have been used extensively as an in vivo small-animal model for studying the pathogenesis of infectious diseases. However, the application of mice for HIV research has been prevented by the inability of HIV to replicate in mouse cells due to blocks at different steps of the HIV replication process, such as the inability of HIV to enter mouse cells because the murine CD4 and CCR5 homologues do not bind to the gp120 envelope glycoprotein (43). The transgenic expression of the human homologue of CD4, CCR5, or CXCR4 by mouse cells relieved the entry block but was insufficient to support sustained HIV replication due to blocks in subsequent replication steps, including the support of HIV-1 Tat function (9, 43).

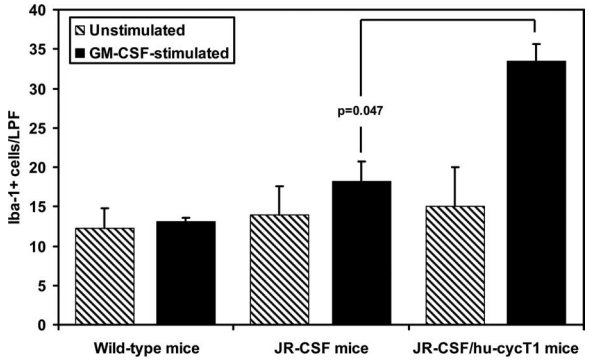

FIG. 10. Quantitative analysis of in vivo microglial activation induced by GM-CSF. Iba-1-positive cells were counted in the cortex region around the hippocampus in four ×100-magnification fields per brain section and are expressed as the average numbers (± standard errors of the means) of Iba-1-positive cells per low-power field of three sections/mouse brain/group (n = 3 or 4 mice/group). The fields evaluated were located in similar regions of the cortex for each section.

Mouse models have been developed to study HIV neuropathogenesis, including mice intracranially injected with HIV-1-infected human cells (55–57) and transgenic mice expressing an HIV-1 gp120 or Tat transgene under the control of a murine GFAP promoter (35, 70). We developed another mouse model to study the pathogenesis of HIV infection, JR-CSF transgenic mice. These mice are transgenic for an infectious full-length HIV-1 provirus derived from the well-characterized primary R5-tropic clinical isolate HIV-1_{JR-CSF} that is under the control of the endogenous HIV-1 LTR, and their T lymphocytes and monocytes produce infectious HIV-1 and display plasma viremia of up to 10^5 HIV copies/ml (10, 51). The LTR-regulated HIV-1 transgene is expressed in the brains and microglia of JR-CSF mice and is responsive to in vivo and in vitro LPS stimulation, and its expression is associated with increased in vivo and in vitro CCL2/MCP-1 gene expression in response to LPS (75). HIV-1 proviral expression and virus production in the JR-CSF transgenic mouse model still were limited by the inability of Tat to function as a transcriptional activator in mouse cells (7, 21, 22). Because HIV replication in JR-CSF mice regulated by the HIV LTR is not specifically

targeted to $CD4^+$ cells, these mice fail to recapitulate many aspects of the pathophysiology of HIV infection. We specifically conferred Tat responsiveness to JR-CSF mouse $CD4^+$ cells by crossing the JR-CSF mice with mice transgenic for the expression of hu-cycT1 under the control of a murine CD4 expression cassette to generate JR-CSF/hu-cycT1 mice. $CD4^+$ T lymphocytes and myeloid-committed cells, but not $CD8^+$ T lymphocytes, from the JR-CSF/hu-CycT1 mice displayed markedly increased in vivo and in vitro HIV-1 production compared to that of JR-CSF mouse $CD4^+$ T lymphocytes and myeloid-committed cells; JR-CSF/hu-cycT1 mouse monocytes displayed increased CCL2/MCP-1 production after GM-CSF and LPS stimulation, and as they aged the JR-CSF/hu-CycT1 mice developed the selective depletion of $CD4^+$ T lymphocytes with a reversal of the ratio of CD4 T lymphocytes to CD8 lymphocytes in the peripheral blood to less than 1 (69). Because HIV production in our JR-CSF/hu-cycT1 mouse model is regulated by the endogenous HIV LTR and selectively increased in cells that express CD4, which is the primary population of infected cells in HIV-1-infected individuals, the JR-CSF/hu-cycT1 mice can be used to study the in vivo regulation of HIV-1 transcription in cells targeted for HIV infection. In the current study, we found increased in vivo HIV and chemokine production associated with increased activation of microglia and astrocytes in the brains of LPS- and GM-CSF-treated JR-CSF/hu-cycT1 mice compared to that of LPS- and GM-CSF-treated control littermate and JR-CSF mice. Therefore, JR-CSF/hu-cycT1 mice may represent an improved model for studying HIV-associated brain pathogenesis and the efficacy of various therapeutic interventions to inhibit HIV-induced neuroinflammation.

HAD is associated with HIVE, which is characterized by the infiltration of monocytes and macrophages into the brain, and the presence of multinucleated giant cells and microglial nodules (12). The brain is populated by three distinct populations of myeloid lineage cells, parenchymal microglia, perivascular and meningeal macrophages, and infiltrating macrophages derived from the peripheral circulation, all of which are infected with HIV-1 in patients with HIVE (14). Microglia play an important role in maintaining neuronal function by taking up excessive neurotransmitters, removing excitotoxins, and eliminating dead cells and cellular debris; this function requires the production of a range of effector molecules that may be neurotoxic, including proteases, oxide radicals, and proinflammatory cytokines (1). Resting microglia express low levels of most surface receptors until activated by interactions with infected or activated cells, viral products, or cytokines/chemokines, which induce the microglia to increase their expression of various cell surface molecules (16). These activated microglia may migrate to areas of the brain in which infection or cellular damage has occurred, secrete cytokines and chemokines, recruit other immune cells, and have the undesired effect of disrupting normal neuronal cell function (41). The extent of monocyte infiltration and microglia activation correlates highly with the development of HIVE and HAD more so than the level of plasma HIV (25, 27, 72), and the number of activated macrophages in the CNS is the most reliable neuropathologic indicator of neurocognitive impairment (25). These findings indicated that the degree to which HIV infection activates resident microglia/macrophages and not the direct effects of HIV infection is the key determining factor in the induction of neurological impairment.

Opportunistic infections with gram-negative bacteria are common in AIDS patients and may lead to increases in local and circulating levels of LPS. In addition, the breakdown of the gut endothelial barrier by HIV infection leads to an increase in plasma LPS levels that activates the innate and adaptive immune systems and likely accelerates the rate of progression to AIDS (8). LPS derived from the gram-negative *Escherichia coli* provides a strong inflammatory stimulus for many different cell types, including monocytes and granulocytes, glial cells, and the brain vascular endothelium (3, 13, 45, 63, 74). LPS binds to CD14 expressed by macrophages and microglia and induces TLR-4 to activate the NF-κB, ERK, and p38 mitogen-activated protein kinase pathways, which can activate the HIV LTR and induce HIV-1 gene expression in infected human myeloid lineage cells (4, 6, 80). LPS induces the synthesis of several potentially cytotoxic factors, cell adhesion molecules (ICAM-1, P-selectin, and E-selectin), and many chemokines, such as MIP-1α, MIP-1β, and MIP-2, that amplify inflammatory responses (23, 26, 64, 66). It is likely that these and other factors induced by LPS combine to activate microglia and astrocytes, as an intracranial injection of tumor necrosis factor alpha alone did not induce comparable cellular activation (data not shown). Systemic LPS does not need to enter the brain to induce inflammation but also can indirectly activate cells in the brain by stimulating cells in the choroid plexus to produce inflammatory cytokines such as tumor necrosis factor alpha, which is secreted into the cerebrospinal fluid and activates brain microglia, resident macrophages, and astrocytes (44). We used the JR-CSF/hu-cycT1 mice to investigate whether systemic LPS synergizes with HIV infection in the brain to activate microglia and astrocytes and to initiate the inflammatory process that leads to HAD. Our study demonstrated that the intracranial injection of LPS more potently induced microglial activation in the brains of JR-CSF/hu-cycT1 mice than in the brains of JR-CSF mice and wild-type littermates, as indicated by quantitative and qualitative Iba-1 staining. Similar results were observed after the systemic administration of LPS, with an almost twofold higher number of Iba-1-positive cells detected in the brains of JR-CSF/hu-cycT1 mice than in the brains of wild-type mice and JR-CSF mice. Thus, the increased levels of LPS in HIV-infected individuals may combine with the dysregulation of microglia function associated with HIV infection to play a direct or indirect contributory role in promoting the neuroinflammatory process that leads to the development of HAD. Treatment with HAART lowered systemic levels of LPS in HIV-infected individuals (8). An intriguing possibility is that HAART lowers the incidence of HAD not only by reducing HIV replication but also by reducing systemic levels of LPS and thereby decreasing the possible contribution of systemic LPS to amplifying HIV-induced neuroinflammation.

Astrocytes, which constitute nearly 40% of the total CNS cell population, play a critical role in maintaining the homeostasis of the brain. The capacity of astrocytes to support HIV replication is limited and is the subject of conflicting reports (11). The production of HIV proteins in infected astrocytes may be induced by treatment with an inflammatory cytokine such as interleukin-1β (65, 71), gamma interferon, or

GM-CSF (11), and these HIV proteins may alter astrocyte function and dysregulate other cells in the brain. Despite the lack of significant HIV production by astrocytes, the HIV-induced activation of astrocytes indicated by GFAP expression is strongly associated with the development of HAD and the severity of disease (76). We observed that after intracranial injection with LPS, the number of GFAP-stained cells was 1.5-fold higher in LPS-stimulated JR-CSF/hu-cycT1 mice than in LPS-stimulated wild-type mice and JR-CSF mice, and JR-CSF/hu-cycT1 mouse brains displayed intense GFAP staining that was associated with the formation of a glial network across all layers of the cerebral cortex. This provides in vivo evidence that HIV infection increases the responsiveness of astrocytes to LPS or factors induced by LPS. These activated astrocytes may release virus proteins, cytokines, and chemokines, including GM-CSF, which may induce a cycle of immune dysregulation and BBB breakdown that allows the entry of increasing numbers of HIV-1-infected monocytes into the brain and the exacerbation of neuroinflammation. CCL2/MCP-1 is the most potent chemokine capable of inducing the migration of monocytes into the brain parenchyma (5, 77, 79). The localized production of CCL2/MCP-1 is sufficient to induce the directed migration of monocytes into organs in vivo, as evidenced by the organ-specific influx of monocytes in mice that carry a CCL2/MCP-1 transgene controlled by an organ-specific promoter (20, 28). By immunohistochemistry we demonstrated that the in vivo administration of LPS stimulated the production of CCL2/MCP-1 by astrocytes more potently in JR-CSF/hu-cycT1 mouse brains than in wild-type and JR-CSF mouse brains. These results indicated that LPS or LPS-induced factors induce HIV-infected astrocytes to produce increased levels of CCL2/MCP-1 and thereby induce the migration of monocytes to the brain.

We demonstrated that sustained systemic treatment with GM-CSF increased the level of HIV production and increased by twofold the number of Iba-1-positive activated microglia in JR-CSF/hu-cycT1 mouse brains. GM-CSF, a cytokine produced rapidly by T cells, monocytes/macrophages, fibroblasts, and endothelial cells in response to infection by a broad range of pathogens, including HIV, can increase HIV production by myeloid lineage cells (78) and also induce the proliferation of microglia (15, 40). Activated astrocytes produce GM-CSF in response to inflammatory signals that would activate microglia, stimulate them to proliferate, and trigger the CNS inflammatory response (41). HIVE is associated with increased GM-CSF production in brains that localized predominantly to astrocytes (15, 67), and the AIDS dementia complex is associated with elevated levels of GM-CSF in the cerebrospinal fluid (53). Systemic GM-CSF was reported to induce a 10-fold increase in the HIV load in the spinal fluid of a treated patient (38), and GM-CSF has been reported to increase HIV-1 replication in brain tissue explants (32) and primary monocyte/macrophages and monocyte cell lines (19, 37, 47, 60). In vivo GM-CSF treatment potently induced HIV-1 production in the spleen and bone marrow of JR-CSF/hu-cycT1 mice (69). Thus, HIV infection may increase GM-CSF production by astrocytes, which then may upregulate HIV production by infected microglia and exacerbate microglial activation and thereby contribute to the pathogenesis of HAD. GM-CSF also may increase the sensitivity of brain myeloid lineage cells to LPS by upregulating TLR-4 expression, as we previously described regarding GM-CSF-treated bone marrow-derived monocytes from JR-CSF mice (51). Taken together, these results indicate that the JR-CSF/hu-cycT1 mice should be a useful model to study various aspects of the in vivo pathogenesis of HAD.

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