The Journal of Immunology

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This information is current as of August 19, 2014.

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J Immunol 2007; 179:4345-4356; ;
doi: 10.4049/jimmunol.179.7.4345
http://www.jimmunol.org/content/179/7/4345

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Copolymer-1 Induces Adaptive Immune Anti-inflammatory Glial and Neuroprotective Responses in a Murine Model of HIV-1 Encephalitis[1]

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Copolymer-1 (COP-1) elicits neuroprotective activities in a wide range of neurodegenerative disorders. This occurs, in part, by adaptive immune-mediated suppression of microglial inflammatory responses. Because HIV infection and immune activation of perivascular macrophages and microglia drive a metabolic encephalopathy, we reasoned that COP-1 could be developed as an adjunctive therapy for disease. To test this, we developed a novel animal model system that reflects HIV-1 encephalitis in rodents with both innate and adaptive arms of the immune system. Bone marrow-derived macrophages were infected with HIV-1/vesicular stomatitis-pseudotyped virus and stereotactically injected into the basal ganglia of syngeneic mice. HIV-1 pseudotyped with vesicular stomatitis virus envelope-infected bone marrow-derived macrophages induced significant neuroinflammation, including astrogliosis and microglial activation with subsequent neuronal damage. Importantly, COP-1 immunization reduced astro- and microgliosis while diminishing neurodegeneration. Hippocampal neurogenesis was, in part, restored. This paralleled reductions in proinflammatory cytokines, including TNF-α and IL-1β, and inducible NO synthase, and increases in brain-derived neurotrophic factor. Ingress of Foxp3- and IL-4-expressing lymphocytes into brains of COP-1-immunized animals was observed. We conclude that COP-1 may warrant therapeutic consideration for HIV-1-associated cognitive impairments. *The Journal of Immunology,* **2007, 179: 4345–4356.**

Persistent in the era of widespread use of antiretroviral therapy (ART),[3] HIV-1-associated cognitive impairments remain a source of significant morbidity in the infected host (1). Neurological complications induced by HIV-1 infection are subtler during ART and often occur without significant immune compromise. Nonetheless, a constellation of motor, cognitive, and behavioral deficits remains clinical hallmarks of disease (1). How cognitive impairments occur in susceptible HIV-1-infected people most likely represents a compendium of genetic determinants, drug compliance, ART resistance, metabolic dysfunctions, viral load in and outside the nervous system, and the inability to clear viral reservoirs coincident with impaired innate and adaptive immune activities (2–5). Nonetheless, disease can occur during the time when the adaptive immune system is functional. This provides a rationale for immune-based therapies for improving disease outcomes during cognitive decline (6).

HIV-1 invades the CNS early following viral infection and the initial seroconversion reaction (7, 8). Evidence abounds that the adaptive immune system can affect viral surveillance and CNS tissue repair (2, 3, 9, 10). T cell-mediated immunity driven to a Th1/2 phenotype can affect microglial activation and induce neurotrophic growth factor release (11–14). T cells also affect neurogenesis and memory formation (15, 16) as well as microglial clearance of misfolded and aggregated proteins in models of Alzheimer's disease (AD) and Lewy bodies in Parkinson's disease (PD) (17–19). This can, in part, be achieved by glatiramer acetate (also called copaxone or copolymer-1), a Food and Drug Administration-approved immunomodulatory drug used for the treatment of multiple sclerosis (20). Copolymer-1 (COP-1) was also shown to be an effective immunomodulatory treatment for neuroprotection in animal models of experimental autoimmune encephalomyelitis, optic nerve crush, PD, and AD (17, 21–23).

Harnessing the immune system to control disease-associated inflammation through COP-1 immunization is an attractive idea to slow the progression of HIV-1-associated cognitive impairments. COP-1 induces Th2 T cell responses secreting anti-inflammatory cytokines, which migrate to the brain and provide bystander suppression against neuroinflammatory and consequent neurotoxic activities (24–27). Based on these prior works, we investigated whether COP-1-mediated anti-inflammatory immune

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Received for publication April 2, 2007. Accepted for publication July 17, 2007.

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[1] This work was supported, in part, by National Institutes of Health Grants 2 R37 NS36126, 1 P01 NS043985-01, 5 P01 MH64570-03, and P20 RR15635.

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[3] Abbreviations used in this paper: ART, antiretroviral therapy; AD, Alzheimer's disease; BDNF, brain-derived neurotrophic factor; BMM, bone marrow-derived macrophage; GFAP, glial fibrillary acidic protein; HIVE, HIV encephalitis; Iba-1, ionizing calcium-binding adaptor molecule 1; i.c., intracranial; iNOS, inducible NO synthase; LN, lymph node; MAP-2, microtubule-associated protein-2; MBP, myelin basic protein; NeuN, neuronal nucleus; NF, neurofilament; PD, Parkinson's disease; PSA-NCAM, polysialylated form of neural cell adhesion molecule; VSV, vesicular stomatitis virus; COP-1, copolymer-1.

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responses could attenuate neuropathological outcomes in rodent models of HIV-1 encephalitis (HIVE). A new animal model for HIVE was adopted in our laboratories based on the ability of pseudotyped HIV to bypass the known viral receptors to infect primary murine cells (28). In C57BL/6 immune-competent mice, bone marrow-derived macrophages (BMM) were injected into the brain after BMM infection using HIV-1 pseudotyped with vesicular stomatitis virus (VSV) envelope glycoprotein (HIV/VSV). In these animals, COP-1 significantly decreased proinflammatory cytokines and inducible NO synthase (iNOS) expression, leading to the protection against neuronal death. This was associated with increased expression of Foxp3 and IL-4. These observations underscore the role of therapeutic immunization as a possible adjunctive therapy for cognitive and motor impairments associated with HIV-1 disease.

Materials and Methods

BMM isolation and HIV/VSV infection

Four-week-old C57BL/6NCrl (C57BL/6) mice were purchased from Charles River Laboratories. Animals were maintained in accordance with critical guidelines for the care of laboratory animals at University of Nebraska Medical Center and National Institutes of Health. Femurs of the mice were excised and flushed with HBSS to obtain bone marrow-derived mononuclear cells. Cells were squeezed through a 40-μm cell strainer to remove the clumps, and enriched on a 60/30 Percoll gradient centrifuged at $500 \times g$ for 30 min. The 60/30 interface was collected, washed, and cultured for 7 days in Teflon flasks at 2×10^6 cells/ml DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, and 2 μg/ml macrophage colony stimulating factor (M-CSF) (a gift from Wyeth, Cambridge, MA) to generate BMM media. Dual-tropic HIV-1_{YU2} and VSV envelope glycoprotein pseudotypes were obtained by cotransfecting plasmids 1 μg of pYU2 and 100 ng of pHIT/G into 293T cells using FuGENE 6 transfection reagent (Roche Diagnostics), as described (29). Pseudotyped virus (HIV/VSV) was collected 48 h posttransfection. Only HIV-1 genes are packaged into the pseudotyped virus, but not the glycoprotein gene of VSV. Thus, the infected cells will produce only HIV proteins. HIV/VSV was used at 1 pg of HIV-1p24/cell to infect BMM. Cells were incubated with virus at 37°C for 6 h, and washed and cultured in complete medium for 5 days to establish the secretion of HIV proteins by mouse macrophages. At day 5 after infection, cells were used to induce HIVE in C57BL/6 mice.

Immune-competent HIVE mice

Four-week-old male C57BL/6 were immunized 1 wk before HIVE induction with 200 μg of COP-1 or saline emulsified in CFA (COP-1 and CFA from Sigma-Aldrich), s.c. at the tail base. To induce HIV encephalitis, syngeneic BMM infected with HIV/VSV pseudotype (5×10^5 cells in 5 μl/mouse) were injected intracranially (i.c.) into the COP-1-immunized or control nonimmunized mice ($n = 12$–14 per group per experiment, wherein 6–7 per group were perfused and used for immunohistology and 6–7 were used for RT-PCR). A total of three experiments was conducted. Sham-operative animals injected i.c. with saline (5 μl) and mice injected with uninfected BMM were used as controls. In replicate experiments, COP-1 was used without adjuvant and administered s.c. at 75 μg/mouse/day in 100 μl of saline starting from 7 days before the induction of HIVE until sacrifice.

Immunocytochemical analyses

Brain tissue was collected at necropsy, fixed in 4% phosphate-buffered paraformaldehyde, and embedded in paraffin. Blocks were cut to identify the injection site. For each mouse, 30 ~ 100 serial (5-μm-thick) sections were cut from the injection site to the hippocampus. Sections were deparaffinized, and immunohistochemical staining followed a basic indirect immunostaining protocol using Ag retrieval by heating to 95°C in 0.01 M citrate buffer for 30 min for all markers, except polysialylated form of neural cell adhesion molecule (PSA-NCAM), which is acid sensitive. On serial sections, immature neurons were localized with Ab to PSA-NCAM (mouse IgM, 1/1000; provided by T. Seki, Jutendo University School of Medicine, Tokyo, Japan). Murine microglia and astrocytes were detected with rabbit polyclonal Abs to ionizing calcium-binding adaptor molecule 1 (Iba-1; 1/500; WAKO) and glial fibrillary acidic protein (GFAP; 1/1000; DakoCytomation), respectively. Abs to neuronal nuclei (NeuN; 1/100), microtubule-associated protein-2 (MAP-2; 1/1000; Chemicon International), and neurofilament (NF, clone 2F11 at 1/100; DakoCytomation) were used to identify neurons. Ab to mouse CD45 was obtained from BD Pharmingen, and the Ab to HIV-1 p24 (1/100) was obtained from DakoCytomation. The polymer-based HRP-conjugated anti-mouse and anti-rabbit Dako EnVision systems are used as secondary detection reagents, and 3,3′-diaminobenzidine (DakoCytomation) was used as the chromogen for all, except for MAP-2 Vector VIP substrate kit (Vector Laboratories) was used. For double staining, primary Abs were applied simultaneously and development steps were performed separately. All paraffin-embedded sections were counterstained with Mayer's hematoxylin. Deletion of primary Abs served as controls. Images were obtained by Optronics digital camera fixed to Nikon Eclipse E800 (Nikon Instruments) using MagnaFire 2.0 software. Quantification of immunostaining was performed using Image-Pro Plus (version 4.0; Media Cybernetics) on serial coronal brain sections.

Ten-micron frozen sections were obtained from brains of animals perfused with peroxidase-lysine-paraforamldehyde fixative (30). Brains were extracted, postfixed in peroxidase-lysine-paraforamldehyde, and saturated with sucrose, and 10-μm sections were cut using a cryostat. Double immunofluorescence staining was performed using Alexa Fluor-488 (green)- and Alexa Fluor-594 (red)-conjugated secondary Abs (Molecular Probes). Laser-scanning images were obtained using a Nikon Swept-field laser confocal microscope (Nikon Instruments).

Real-time RT-PCR

Total RNA from brain sections (2 mm thick) containing the injection line was extracted using TRIzol method (Invitrogen Life Technologies). RNA was extracted using RNA mini columns (Qiagen), and reverse transcribed to cDNA with random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). Real-time quantitative PCR was performed with cDNA using an ABI PRISM 7000 sequence detector (Applied Biosystems). Murine-specific primer pairs were as follows: GFAP, 5′-ACTGGGTACCATGCCACGTT-3′ and 5′-GGGAGTGGAGGAGTCATTCG-3′; CD11b, 5′-GCCAATGC AACAGGTGCATAT-3′ and 5′-CACACATCGGTGGCTGGTAG-3′; IL-1b, 5′-CTATGCTGCCTGCTCTTACTGACT-3′ and 5′-CGGAGCC TGTAGTGCAGTTG-3′; IL-6, 5′-TTCCATCCAGTTGCCTTCTTG-3′ and 5′-GAAGGCCGTGGTTGTCACC-3′; and iNOS, 5′-GGCAGCCT GTGAGACCTTTG-3′ and 5′-GAAGCGTTTCGGGATCTGAA-3′. A SYBR Green I detection system was used, and quantification was done using standard curve method, as described in User Bulletin 2 obtained with ABI PRISM 7000 sequence detector. TNF-α, BDNF, IL-4, CD3, and Foxp3 expressions were analyzed using TaqMan gene expression assays. Gene expression was normalized to GAPDH. All PCR reagents were obtained from Applied Biosystems.

Cytokine analysis

Lymph node (LN) cells were plated in 96-well plates at 2×10^5 cells/well/200 μl. Cells were cultured in vitro with Con A (1 μg/ml; Sigma-Aldrich), COP-1 (15 μg/ml), myelin basic protein (MBP; 15 μg/ml), or HIV-1_{BAL}gp120 protein (0.5 μg/ml; National Institutes of Health AIDS Reagent Program). After 72 h, 100 μl of cell suspension was collected for cytokine analysis, and supernatants were analyzed by cytokine bead arrays (BD Biosciences) for Th1/Th2 cytokines and inflammatory cytokines, including IL-4, IFN-γ, IL-10, and IL-12p70. The assay was performed in duplicate and included standards for each cytokine following manufacturer's instructions. The beads were analyzed on a BD FACSCalibur using BD CellQuest Software.

Statistical analyses

The results were expressed as mean ± SEM for each group with six to seven animals. Statistical significance between groups was analyzed by Student's t test and ANOVA using SAS software (V9.1). Two-way ANOVA was used to compare multiple groups. Pairwise comparisons were performed using Tukey's method. Differences were considered statistically significant at $p < 0.05$.

Results

Development of a murine HIVE model to study immune-based therapies

To evaluate the role of adaptive immune system for therapeutic immunization and neuroprotection, a new murine model of HIVE was developed (Fig. 1). This animal model permits the study of both innate and adaptive immune responses to immunization. An

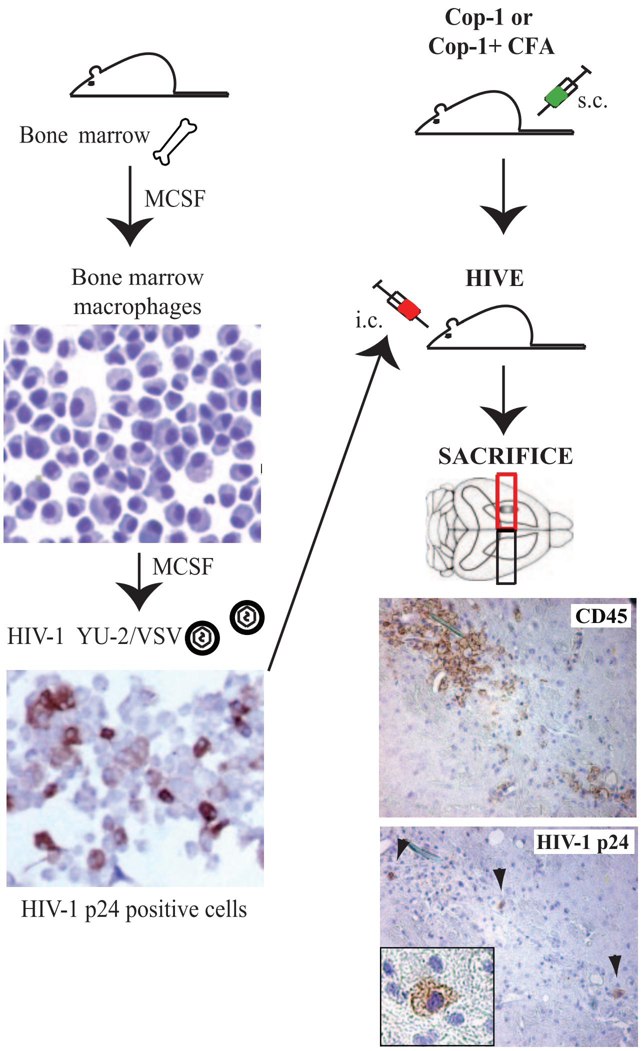

FIGURE 1. Schematic diagram of the HIVE mouse model. Mice were injected i.c. with HIV/VSV-infected syngeneic BMM. BMM were obtained by cultivating bone marrow cells in M-CSF for 7 days following HIV/VSV infection. HIV-1p24 staining of cytospin preparations of infected BMM (day 5 after infection) is shown in the figure. One week before injecting infected BMM into the mouse brain, mice were immunized with COP-1 (200 μg/mouse) in CFA s.c. at the tail base. We also used a different immunization regimen in which animals were injected with COP-1 without CFA (75 μg/mouse) s.c. everyday from 7 days before HIV-infected BMM injection until sacrifice. Brain tissues were collected at necropsy, fixed in paraformaldehyde, and processed for paraffin embedding. Sections (5 μm) containing the injection area were cut, then stained immunohistologically for CD45, to detect injected BMM and for leukocytes recruited to the site of encephalitis. HIV-1-infected BMM were detected with HIV-1 p24 Ab. Serial sections stained for CD45 and HIV-1 p24 are shown at the original magnification of ×200, with an *inset* of the HIV-1 p24-positive cell marked with the white arrow, shown at ×600.

immune-deficient HIVE rodent model was established in our laboratory by injecting HIV-1-infected human macrophages into the basal ganglia (31). These animals are devoid of functional T and B cells, and as such, inadequate to study T cell-based immune therapies for HIVE. In the present model, murine BMM infected with HIV-1/VSV-pseudotyped virus were injected into the brains of syngeneic C57BL/6 mice with intact adaptive immune system (Fig. 1). BMM were used as a source of macrophages, and HIV-1-infected BMM are injected into the brain to induce encephalitis, because macrophage population is the principal infected cell in human HIV encephalitis. HIV-1 pseudotyped with the VSV envelope glycoprotein has been shown to productively infect murine astrocytes, lymphocytes, and macrophages (28, 32).

Histopathological observations of brain tissues around the injection line showed lymphocyte infiltration representing the engagement of the adaptive immune system. Ab to mouse CD45 identified BMM (CD45^{low}-expressing cells) and infiltrating leukocytes (CD45^{high}-expressing cells). Leukocytes that had infiltrated the brain were found in and around BMM-injected area (Fig. 1), and were readily identified as small darkly stained cells. HIV-1 p24 immunostaining of brain sections showed very few HIV-1-infected BMM, <5% of total cells injected (Fig. 2). This may be due to faster clearance of infected cells given an intact immune system in the immune-competent mice. However, significantly higher levels of microglial activation and astrogliosis were observed in mice injected with HIV-1-infected BMM when compared with uninfected BMM or sham controls (Fig. 2).

Two different immunization regimens were followed. In our first set of experiments, we immunized animals with a single injection of COP-1 emulsified in CFA. In parallel experiments, we injected COP-1 by s.c. route everyday, which could be applied for human use. COP-1 immunization is referred to in text as either everyday drug injection or when administered once with CFA.

COP-1-induced adaptive immune responses

Peripheral immune responses to COP-1 were studied to assess whether immunization with a weak agonist of a self-Ag could simultaneously induce both Th1 and anti-inflammatory Th2-polarized T cell responses in HIVE mice. Pooled LN cells from sham-injected or HIV/VSV HIVE animals either immunized with COP-1-CFA (HIVE + COP-1) or saline-CFA controls (HIVE) were cultured with COP-1, MBP, or HIV-1gp120. Cytokine bead array analyses demonstrated increased levels of IL-10 and IL-4, and decreased levels of IL-12p70 by LN cells of COP-1-immunized mice compared with nonimmunized and sham mice (Fig. 3). COP-1 stimulation significantly triggered IL-10 production. Importantly, COP-1 immunization did not decrease IFN-γ secretion by LN cells from mice with HIVE in response to HIV-1gp120.

COP-1 modulates glial activation in HIVE mice

To determine the effect of COP-1 immunization on glial activation in HIVE mice, the numbers of BMM in the injection site and extent of gliosis were assessed by immunohistochemistry from brains extracted at day 7 after HIVE. BMM present in the mouse brain was quantified by immunostaining with mouse CD45 Ab. Stained cells per section were quantified as percentage of stained area of the total field of view, and the average was determined from three sections per mouse and six mice per group. Numbers of CD45$^+$ cells as indicated by the area of CD45 staining were not significantly different in either COP-1-CFA-immunized or saline-CFA-injected HIVE groups (Fig. 4). Astrogliosis and microglial activation were determined as a measure of the intensity of GFAP and Iba-1 expression. Widespread astrogliosis observed by increased levels of GFAP immunostaining around the injection site of BMM in saline control mice was significantly reduced by COP-1 immunization. Similarly, the intensity of Iba-1 expression by activated microglial cells was significantly ($p < 0.02$) diminished, almost 2- to 3-fold, in COP-1-immunized brains compared with controls (Fig. 4).

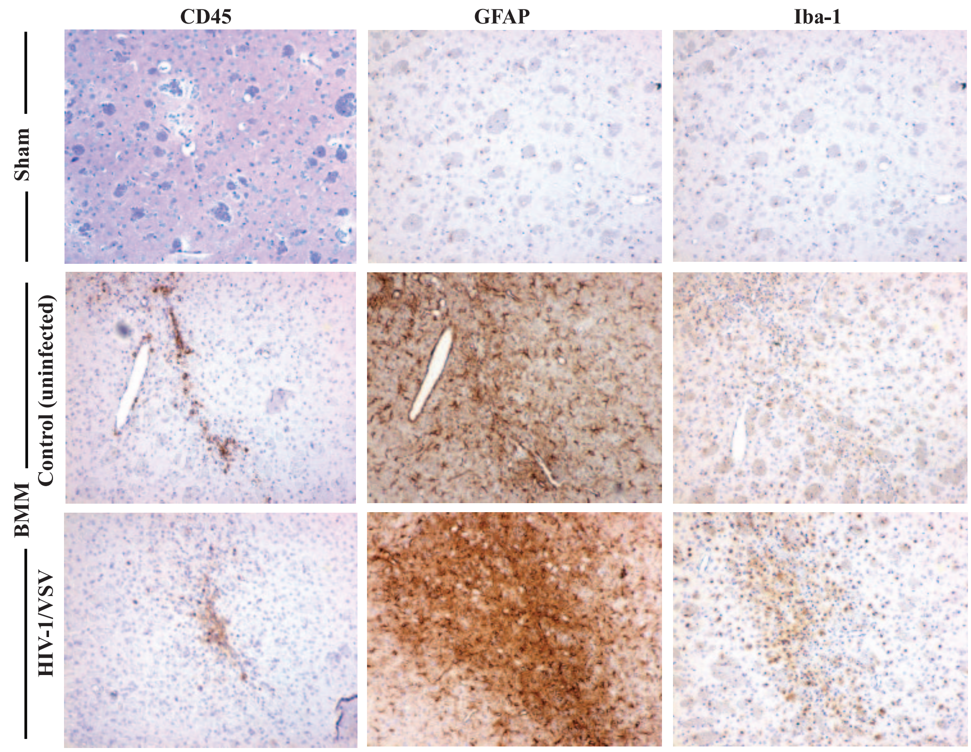

FIGURE 2. HIV/VSV-infected BMM in brain induces HIVE pathology. Neuropathological effects of virus-infected BMM in brains of immune-competent mice were assessed. Immune-competent mice were stereotactically injected with either HIV/VSV-infected BMM or uninfected BMM, and serial sections of brain tissue, including the injection area, were obtained and analyzed by immunocytochemical assays for CD45, GFAP, and Iba-1 Ags. Sham-operated mice were injected with 5 μl of PBS. Brains were collected at day 7 after intracranial injections. Representative photomicrographs are shown at the original magnification of $\times 100$ for CD45, GFAP, and Iba-1. Profound neuroinflammation as determined by GFAP and Iba-1 immunoreactivity was observed in HIV/VSV BMM-injected animals when compared with sham or uninfected BMM.

COP-1-induced neuroprotection in HIVE mice

Neuronal integrity was analyzed by MAP-2 and NeuN staining of serial brain sections from HIVE and COP-1-immunized HIVE animals (with or without CFA; Figs. 5 and 6, respectively). The results showed the loss of MAP-2 and NeuN staining extended beyond the injected area in nonimmunized saline control animals. COP-1 immunization increased the area of MAP-2 immunoreactivity around the injection site compared with controls, thus effectively reducing the lesion size in COP-1-immunized mice (Fig. 5; $p < 0.01$). The larger lesion size in nonimmunized animals may be due to increased gliosis around the injection site. However, the intensity of MAP-2 around the lesion is significantly lower in nonimmunized animals when compared with COP-1-immunized brains, which is shown under higher magnification (Fig. 5). Ab to NF that recognizes phosphorylated and nonphosphorylated forms was used to identify neuronal cell bodies undergoing degeneration. Phosphorylated heavy NF subunits are normally restricted to axons, but in trauma they are shown to accumulate in neuronal perikarya, which are normally devoid of phosphorylated subunits (33); thus, degenerating neuronal cell bodies are shown as darkly stained NF-immunoreactive cell bodies. NF staining showed a significant number of degenerating neurons in the BMM injection site of nonimmunized mice, whereas these degenerating neurons were scarcely seen in the injected areas of COP-1-immunized animals (Fig. 5). Moreover, numbers of NF-immunoreactive cells were significantly reduced in COP-1-immunized HIVE mice compared with controls ($p < 0.005$).

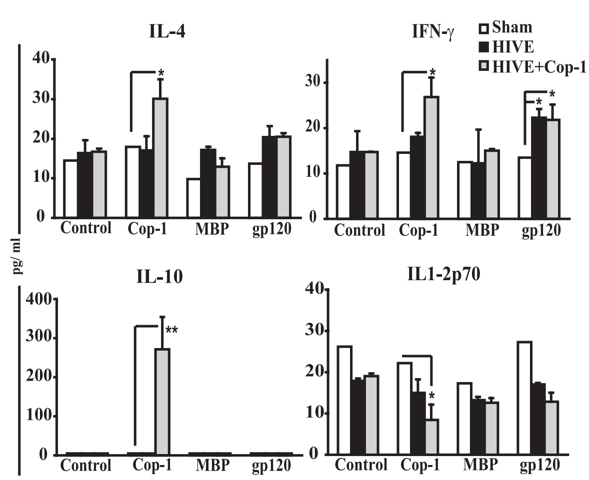

FIGURE 3. Peripheral immunity to COP-1 and HIV-1gp120 Ags. LN were collected from mice treated with saline i.c. (Sham, □), HIV/VSV-infected BMM i.c. and immunized with saline-CFA (HIVE, ■), or HIV/VSV-infected BMM i.c. and immunized with COP-1-CFA (HIVE + COP-1, ▨). Lymphocyte isolates were stimulated with COP-1, MBP, and HIV-1gp120, or were cultured with medium alone (control). Supernatants were collected at 72 h after stimulation and analyzed for Th1/Th2 and inflammatory cytokines using cytokine bead arrays. LN from two animals in each group were pooled to obtain lymphocytes for culture, and the values are expressed as mean ± SEM of three pooled samples per group ($n = 6$). Differences between treatments were assessed by ANOVA, and pairwise differences were determined by Tukey's posthoc tests. *, $p < 0.05$; **, $p < 0.01$ compared with sham controls stimulated with each respective Ag.

To more closely simulate a clinical therapeutic regimen for COP-1 that could be used in infected humans, C57BL/6 mice were treated daily with 75 μg of COP-1 beginning at 7 days before i.c. injection of HIV/VSV-infected BMM and continuing until sacrifice. Confocal fluorescent images of NeuN/MAP-2 staining from HIVE mice indicated that neuronal integrity was preserved in COP-1-immunized animals with reduced lesion size (Fig. 6). Additionally, immunofluorescence staining showed expression of the neurotrophic protein, BDNF. BDNF was significantly increased in COP-1-immunized HIVE mice exceeding levels of expression in untreated HIVE mice (Fig. 6). BDNF-positive staining was observed in the nucleus and cytoplasm, supporting what was shown previously (34). The sections were also stained for GFAP along with BDNF, in which GFAP-positive staining

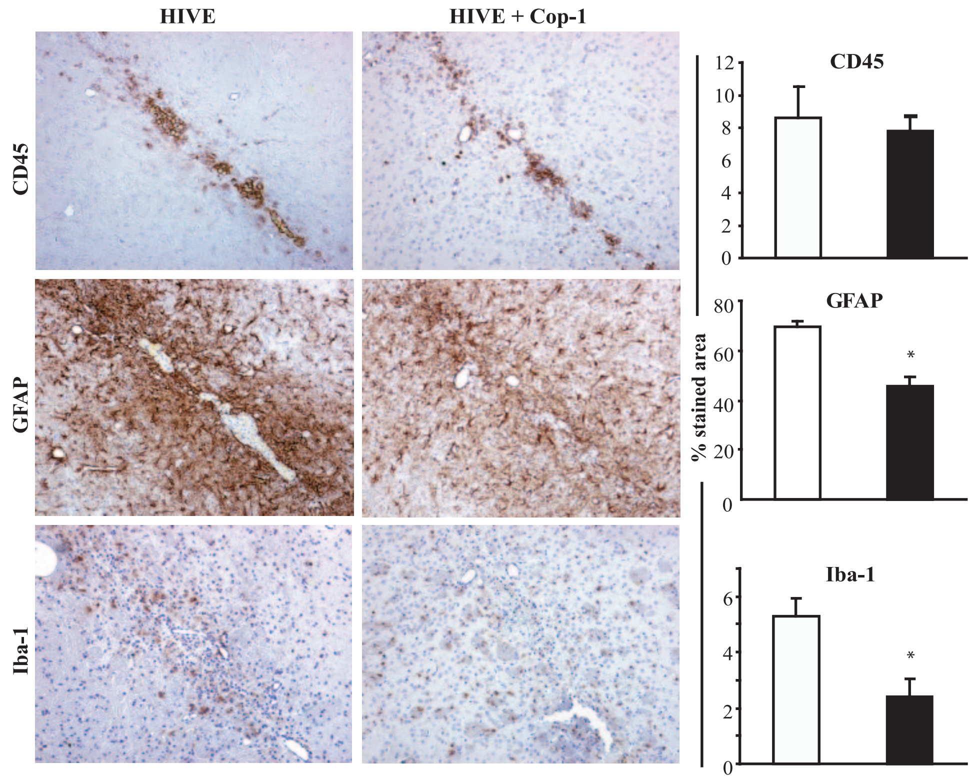

FIGURE 4. COP-1 treatment modulates neuroinflammation in HIVE animals. Serial sections of brains from HIVE mice immunized with either saline-CFA (HIVE) or COP-1-CFA (HIVE + COP-1) were stained for CD45, GFAP, and Iba-1 (magnification, ×100). Stained areas were digitally analyzed, quantified, and expressed in bar graphs as mean percentage of area stained positive in a field of view. Values are expressed as mean ± SEM for six animals per group, and the value for each animal was averaged from three stained slides per animal. Means from control groups (□) were compared with COP-1-immunized groups (■) by Student's *t* test; *, $p < 0.05$.

is notably reduced in COP-1-immunized HIVE mice when compared with nontreated HIVE controls.

To confirm these results, we evaluated the gene expression by RT-PCR, using RNA extracted from ipsilateral and contralateral hemispheres of COP-1-immunized and nonimmunized HIVE mice. The results indicated a significant down-regulation of GFAP expression in ipsilateral injected area of COP-1-immunized immunocompetent mice (Fig. 7, $p < 0.01$). Significant reduction in microglial activation in the ipsilateral hemisphere, as detected by Iba-1 staining (Fig. 4), was confirmed by RT-PCR for CD11b expression (Fig. 7, $p < 0.05$). RT-PCR analysis for proinflammatory cytokines in the ipsilateral hemisphere of COP-1-immunized HIVE mice revealed a significant down-regulated expression of TNF-α, IL-1β, and IL-6, proinflammatory cytokines shown to affect neuronal integrity. Additionally, the reduction in proinflammatory cytokines coincided with reduced

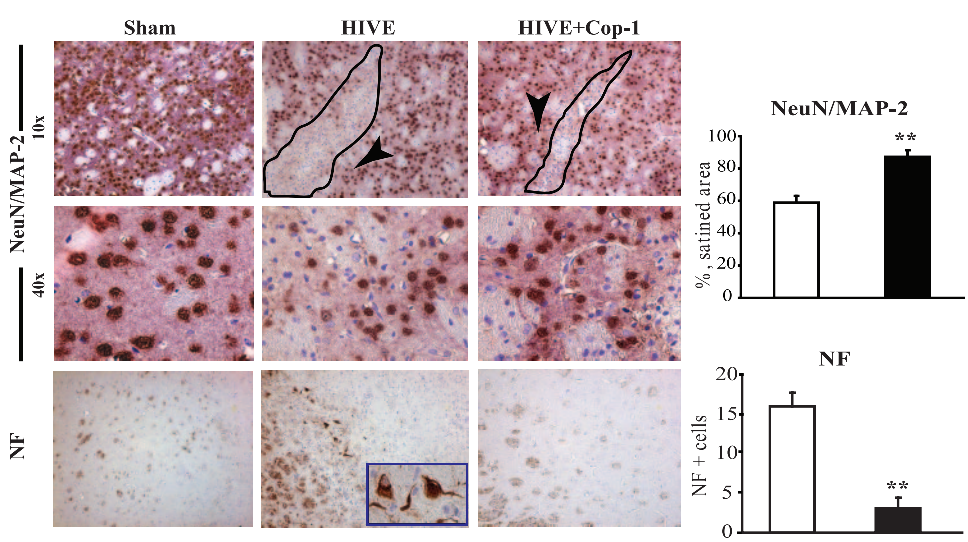

FIGURE 5. COP-1-induced neuroprotection in HIVE mice. Paraffin-embedded serial sections of brains from sham-injected mice and HIVE mice immunized with either saline-CFA (HIVE) or COP-1-CFA (HIVE + COP-1) were stained for NeuN (brown) and MAP-2 (purple), or NF (original magnification ×100). The outlined area surrounding the injection site represents the areas of neuronal loss. Magnified views of brain regions adjacent to the injection line (marked with arrowheads) are shown in the *bottom panels*. Accumulation of NF in the neuronal bodies of injured dying neurons is shown in the *inset* (original magnification ×400). NeuN/MAP-2-stained sections were digitally analyzed, quantified, and expressed in bar graphs as mean percentage of area stained positive in the field of view. NF-positive neuronal bodies were counted manually. Values are expressed as mean ± SEM for six animals per group, and the value for each animal was averaged from three stained slides per animal. Means from control groups (□) were compared with COP-1-immunized groups (■) by Student's *t* test; *, $p < 0.005$.

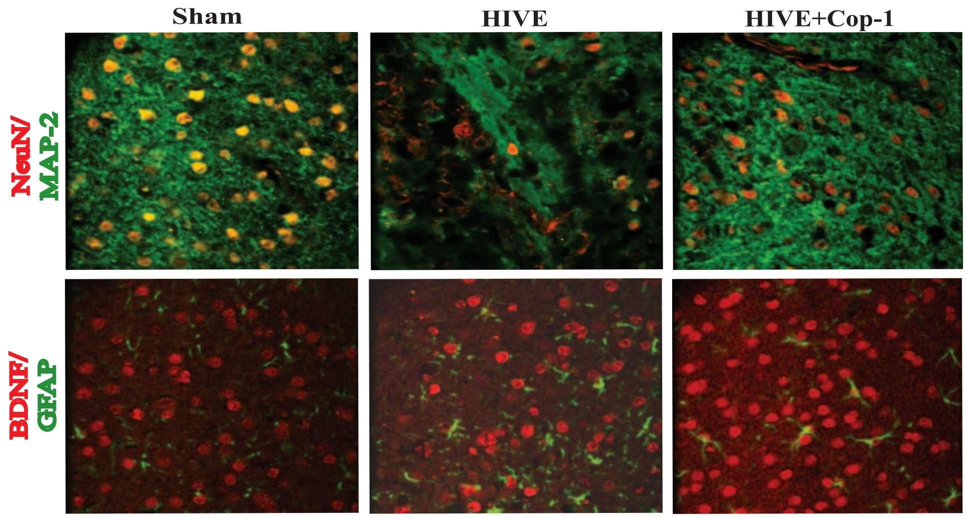

FIGURE 6. COP-1 induces BDNF expression in HIVE mice. Frozen sections of brains obtained from animals that were injected i.c. with saline (Sham), injected i.c. with HIV/VSV-infected BMM (HIVE), or injected i.c. with HIV/VSV-infected BMM and immunized with daily injections of COP-1 without adjuvant (HIVE + COP-1). Sections were stained by primary Abs for expression of NeuN (red), MAP-2 (green), GFAP (green), and BDNF (red). Alexa Fluor-488 (green)- and Alexa Fluor-594 (red)-conjugated host-specific secondary Abs were used to detect the primary Abs, and tissues were visualized by confocal laser-scanning microscopy. Images are shown at ×400 magnification.

expression of iNOS in both ipsilateral and contralateral hemispheres of COP-1-treated animals.

T cells and neurotrophins

Immunostaining of brain sections with CD45 showed the presence of CD45^{high} lymphocytic cells (arrows) in close proximity to CD45^{low} BMM (Fig. 8); the former also differentiated from BMM by smaller size and more intense expression of CD45 compared with BMM. Because levels of T cells were not sufficient to yield statistically reliable numerical estimates, quantification of infiltrating T cells was determined by quantitative RT-PCR for CD3 (35, 36). CD3 RT-PCR showed an increased lymphocyte infiltration in nonimmunized animals (Fig. 8) that may be due to greater microglial inflammation in these animals compared with COP-1-immunized brains. Foxp3 (preferentially expressed by regulatory T cells) and IL-4 were analyzed by RT-PCR for the presence of Th2 immune cells in the brain of COP-1-immunized mice. A significantly greater ratio of Foxp3

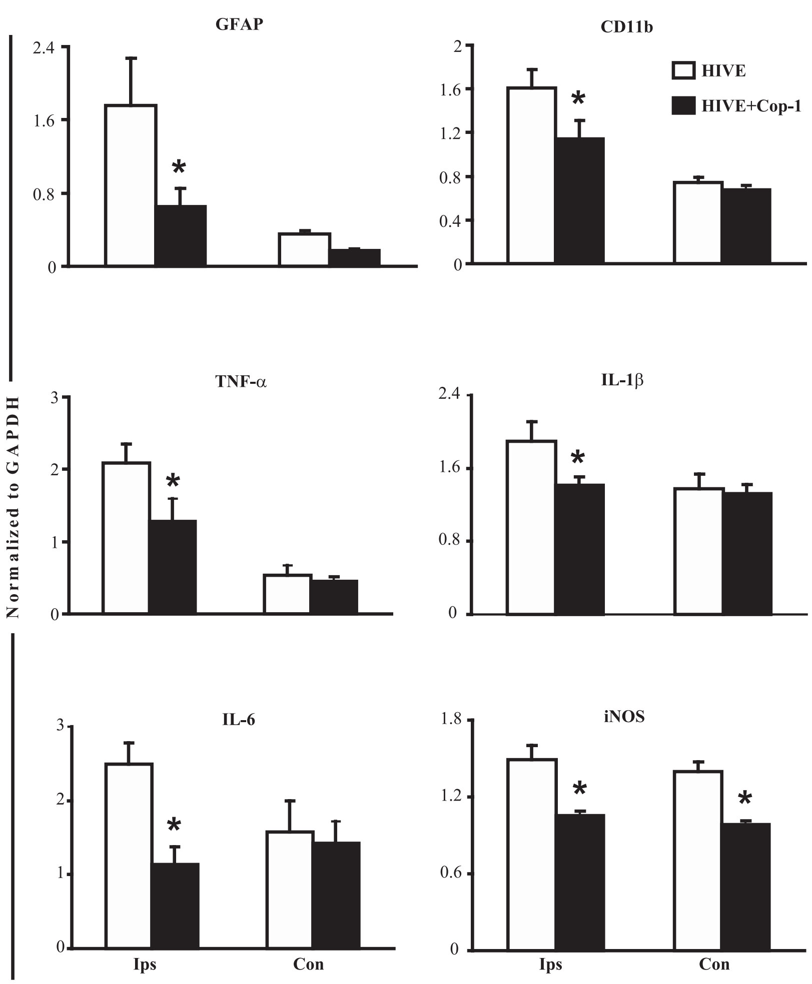

FIGURE 7. RT-PCR for the assessment of inflammation and proinflammatory cytokine expression. Expression of RNA encoding inflammation markers and proinflammatory cytokines such as GFAP, CD11b, TNF-α, IL-1β, IL-6, and iNOS was assessed by RT-PCR. RNA was prepared from the ipsilateral (Ips) and contralateral (Con) hemispheres of mice injected with HSV/VSV BMM and saline-CFA (HIVE, □) or COP-1-CFA (HIVE + COP-1, ■). The gene expression was normalized to GAPDH used as an endogenous control. Normalized values are expressed as mean ± SEM from six animals per group, and compared by Student's t test; *, $p < 0.05$.

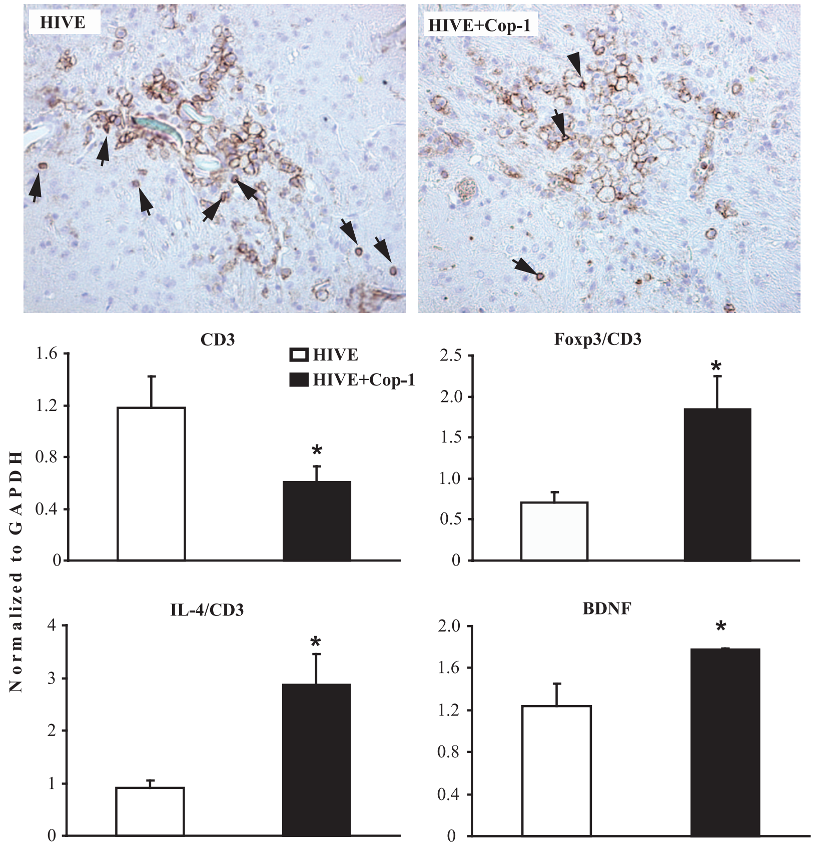

FIGURE 8. T cells ingress in brains of HIVE animals. Brain sections of HIVE saline-CFA (HIVE)- and COP-1-CFA-immunized animals (HIVE + COP-1) stained for CD45 showing the injection site of HIV/VSV-infected BMM and lymphocytes (marked with arrowheads) as smaller, more intensely stained brown cells (magnification ×200). Bar graphs represent real-time PCR for CD3, Foxp3, IL-4, and BDNF expression within the injected hemisphere of HIVE mice that were nonimmunized (HIVE, □) or immunized with daily COP-1 injections (HIVE + COP-1). Gene expression was normalized to GAPDH, and Foxp3 and IL-4 were further normalized to the expression of CD3. Mean values ± SEM were calculated from six animals per group, and *, $p < 0.05$ compared with nonimmunized control by Student's *t* test.

and IL-4 mRNA expression to total CD3 expression was detected in COP-1 animals compared with nonimmunized animals (Fig. 8), suggesting the presence of Th2-polarized lymphocytes in the injected area of COP-1-immunized animals. Additionally, BDNF mRNA expression was found to be significantly increased (by 33%, $p < 0.05$) in the brains of COP-1-immunized animals relative to nonimmunized HIVE animals (Fig. 8), thus confirming the increased expression of BDNF protein by immunofluorescence (Fig. 6).

COP-1 affects neurogenesis

Because COP-1 is known to affect neuronal regeneration in experimental autoimmune encephalitis (15), we next evaluated the effects of COP-1 on neuronal progenitor cells in HIV/VSV-induced HIVE. Previous work in our laboratory demonstrated that proliferation of progenitor cells is reduced in the hippocampus of C.B-17, C.B-17/*scid*, and NOD/*scid* mice during HIVE induced by HIV-1-infected human macrophages (37). To assess the capacity of COP-1 to support hippocampal neurogenesis in HIVE, we stained hippocampal subregions for the expression of PSA-NCAM. HIVE generated from HIV/VSV-infected cells injected into the basal ganglia reduced the intensity of PSA-NCAM staining in the dentate gyrus of C57BL/6 mice compared with sham-operated controls (Fig. 9). This was confirmed by significant reductions in both the number of PSA-NCAM-positive cells and the dendritic lengths of HIVE animals compared with sham-injected

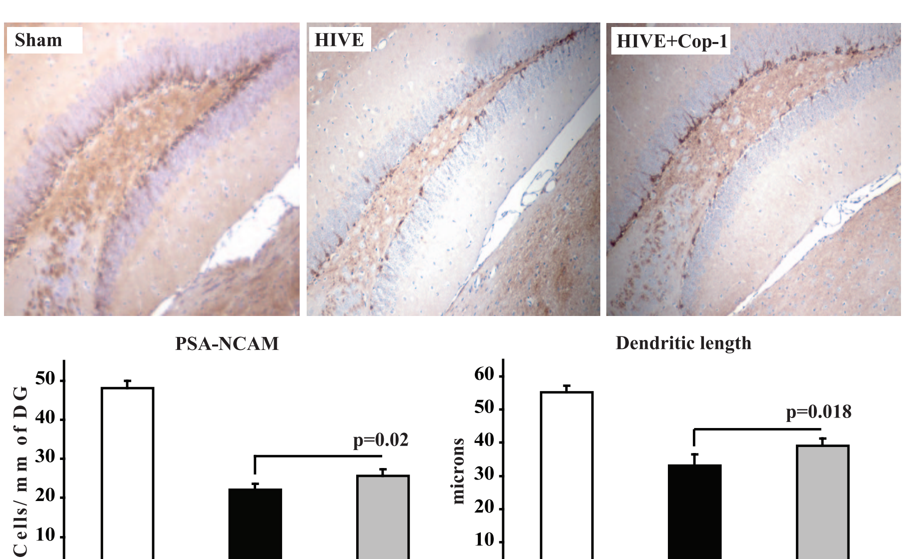

FIGURE 9. COP-1 immunization restores neurogenesis in hippocampus of HIVE animals. Expression of PSA-NCAM (brown) was determined by immunohistochemistry in hippocampi of mice treated with saline i.c. (Sham), HIV/VSV-infected BMM i.c. and immunized with saline-CFA (HIVE), or HIV/VSV-infected BMM i.c. and immunized with COP-1-CFA (HIVE + COP-1) (magnification ×200). Numbers of PSA-NCAM-immunoreactive cells (PSA-NCAM) and length of dendrites (dendritic length) were determined in sham controls (□), saline-CFA-immunized HIVE (■), and COP-1-immunized HIVE (▩) mice. Means ± SD were calculated from six mice per group. Significance between immunized and nonimmunized animals was determined by Student's *t* test.

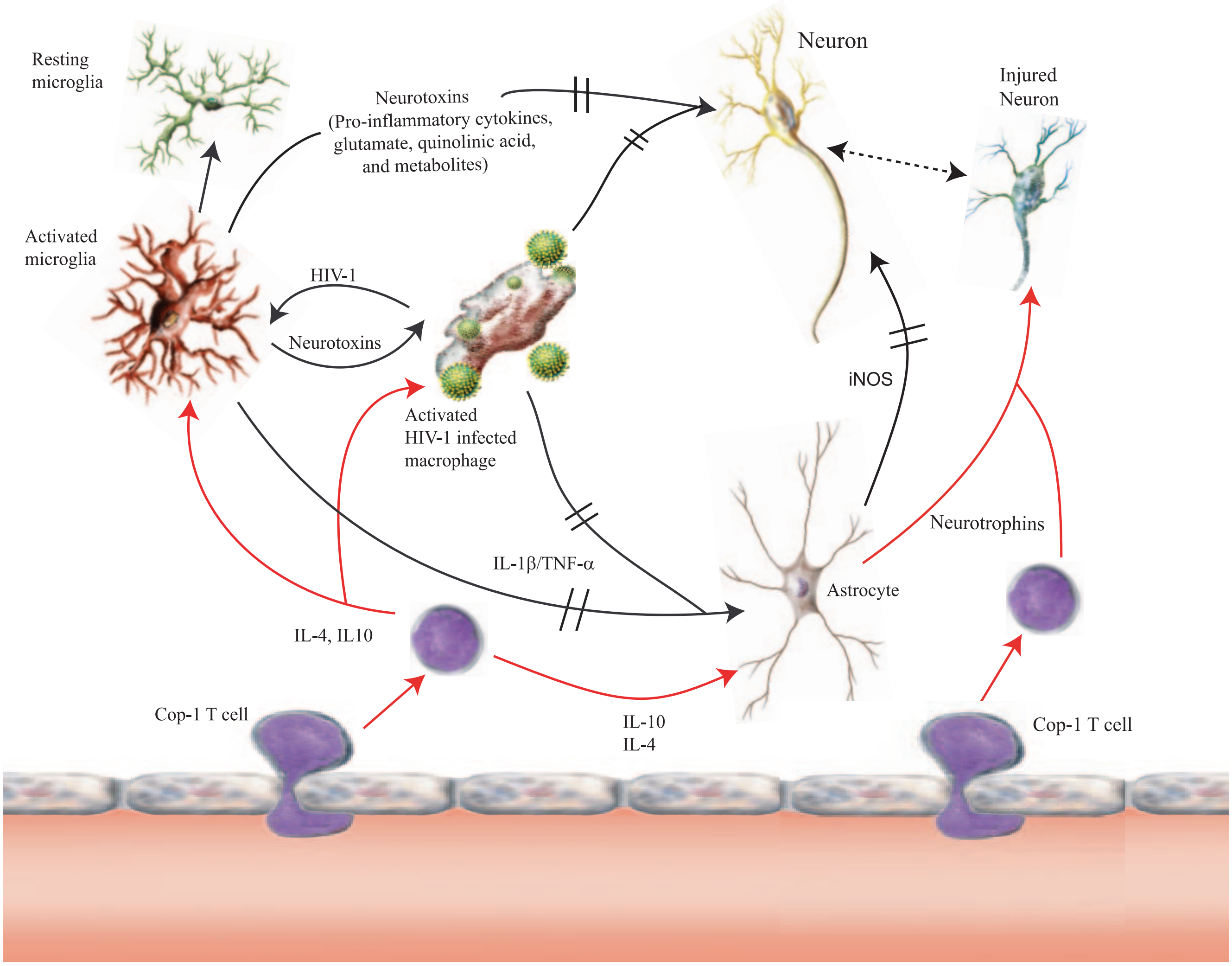

FIGURE 10. Mechanisms for COP-1-induced neuroprotection and HIVE. After transendothelial migration of HIV-1-infected MDM through an affected blood brain barrier, differentiated brain macrophage and the indigenous microglia serve as vehicles for viral dissemination throughout the brain and incite a metabolic encephalopathy. Macrophage and microglia neurotoxic secretions include a variety of immunoregulatory factors (proinflammatory cytokines, chemokines, platelet-activating factor, quinolinic acid, glutamate, and, to a lesser extent, NO), which affect neural and glial function leading to CNS inflammation. This immunoregulatory response inevitably becomes amplified (by autocrine and paracrine mechanisms) and ultimately leads to neurodegeneration. Following COP-1 immunization, Th2 T cells are induced and infiltrate into the inflamed areas of the brain, where they encounter cross-reactive self-Ags such as MBP presented in the context of MHC by resident microglia. These lead to anti-inflammatory activities, following the release of macrophages and T cell IL-10 and IL-4 cytokines, which suppress glial inflammatory and neurotoxic activities. T cells may directly secrete neurotrophins or T cell-derived IL-4 and IL-10, and macrophage-derived IL-10 may induce neurotrophin production in neighboring glia. COP-1 immunity leads to neuroprotection indirectly by suppression of microglial responses and directly by providing neuronal nutritive support through neurotrophin release.

mice. COP-1 immunization statistically increased the number of neuronal progenitor cells with a concomitant effect in dendritic length by 10–15%.

Altogether, these results suggest and support the previous observations that COP-1 acts through induction of COP-1-specific Th2 responses. Fig. 10 demonstrates how COP-1 may lead to neuroprotection during HIVE. The overview includes proposed mechanisms of T cell-mediated anti-inflammatory activities, deactivation of glial immune responses, and the role of autoantigen-specific adaptive immunity in neuroprotection.

Discussion

HIV-1-associated cognitive and motor impairments are characterized by secretory factors produced from immune-competent and virus-infected perivascular macrophages and microglia eliciting a metabolic encephalopathy (38). Neural injury in disease is induced by viral and cellular factors from immune-competent perivascular macrophages and microglia (39–41). These factors include, but are not limited to, the HIV-1 proteins such as gp120 and tat, proinflammatory cytokines, arachidonic acid and its metabolites, quinolinic acid, and glutamate (42–44). Prior works performed in animal models and in humans have shown that adjunctive microglial immune modulatory drugs can affect HIV-1-associated cognitive decline (43, 45–47). Such neuroprotective therapies interrupt direct damage to neurons by attenuating the effects of macrophage activation.

Neuropathological observations of myelin pallor, multinucleated giant cell formation, astro- and microgliosis, and neuronal loss, features of advanced HIV-1 infection and encephalitis, are associated with cognitive, motor, and behavior dysfunctions (48–52). Nonetheless, HIV-associated mild cognitive impairments still persist, despite the widespread use of antiretroviral therapy, and can occur throughout the course of viral infection (53). HIV-1-infected people often show impaired psychomotor speed, spatial and verbal memory, and fine motor control (54–57). These observations parallel those seen in animal models of neuroAIDS. Indeed, SIV-infected rhesus macaques demonstrate neural dysfunction in the early stages of viral infection (58, 59). Interestingly, such observations parallel T cell ingress to brain and neuronal functional disturbances (58, 60). Although the presence of virus-specific T cells in the brain is crucial for elimination of viral infection, T cells accumulate in brains of SIV-infected monkeys even after the elimination of virus. This gives rise to the question of under what circumstances is accumulation of T cells in the brain harmful or helpful? Activated proinflammatory Th1-directed T cells infiltrate the brain during HIV infection and could perpetuate the mononuclear phagocyte (perivascular macrophage and microglia) responses, and as such could accelerate neuronal dysfunction and deficits in neural structural integrity. This is supported by the fact that nervous system dysfunction can occur throughout the course of viral infection associated with ongoing vigorous immune responses. On balance, the presence of T cells in brain at early stages of SIV infection (58) and in humanized HIVE mice (10) suggests that modulation of T cell function could also provide a neuronal protective response for disease.

The role of T cells in the neuropathogenesis of HIV-1 infection is supported by a number of recent reports. T lymphocytes were found in HIV-1-infected brain tissues. Although such accumulation is minor, it is nonetheless consistent (61). Although $CD4^+$ and $CD8^+$ T cells are both present, more attention has been paid to the latter due to its antiretroviral activities. $CD8^+$ T lymphocytes have been shown to specifically traffic into the nervous system in an angiocentric and Ag-specific manner (62). Indeed, numbers of $CD3^+$ and $CD8^+$ T cells are significantly increased in perivascular spaces and inflammatory nodules during HIVE, but are rare or absent in HIV-1-infected brains without encephalitis. HIVE brains contain granzyme B^+ T cells and express perforin. CTLs directly contact with neurons. These are rare or absent in patients who died of HIV disease, but had no evidence of encephalitis, (63). CTL could affect brain injury in HIVE, and as such, could be a biomarker for productive HIV-1 infection in brain in disease. In regard to chronic infection, 2 years after SIV infection of rhesus macaques, nervous system dysfunction is readily demonstrated. In these animals, infiltrating $CD8^+$ T cells were found in the brain (64). For feline immunodeficiency virus infection of cats, brain disease is characterized by infiltrates of $CD79^+$ and $CD3^+$ B and T cells (mixture of $CD4^+$ and $CD8^+$ cells). The severity of lesions for feline immunodeficiency virus infection increases in intensity in weeks and is comparable to what is seen in the early stages of HIVE (65). In human HIV disease, antiretroviral therapy can affect immune restoration, and neuropathological examinations have shown severe inflammatory and/or demyelinating lesions linked to intraparenchymal and perivascular infiltration by T lymphocytes (66). These T cells are commonly $CD8^+$. In those cases with a lethal outcome, inflammation was severe. Fulminant leukoencephalitis can result from T cell dysregulation (67). In contrast, infiltration of $CD8^+$ T cells in the brain is commonly associated with immune activation markers and HIV-infected cells and genetic segregation of brain variants from populations in lymphoid tissues. $CD8^+$ T cells can limit replication of HIV in the nervous system. The prevailing view is that neurological complications that result from progressive HIV disease evolve when T cell control mechanisms break down after progressive immunosuppression characterized, in part, by the destruction of $CD8^+$ T cells (68). These results, taken together, suggest that compromise of the adaptive immune system heralds the development of the neurological complications of viral infection. How such cells affect disease progression is certainly incompletely understood. Indeed, extensive pathological studies have failed to demonstrate T cell links to neuropathological endpoints (48–52, 69, 70).

Immune-deficient mice injected with HIV-infected human macrophages into the subcortex serve as a model for the studies of HIVE and were developed in our laboratories (10, 31). However, the lack of long-lived adaptive immune component in these mice renders this model deficient to investigate immune-based therapies. Thus, a novel immunocompetent HIVE mouse model was used in the present study. The strengths are as follows. First, encephalitis can develop in a murine system with murine macrophages expressing viral proteins. Second, immunity can be studied in regard to the progression of HIVE. Indeed, the model is a conceptual advance because it allows bimodal study of both innate and adaptive immune responses that follow viral infection of macrophages in the brain and consequent encephalitis, as reported elsewhere (71). Third, bone marrow macrophages may more adequately reflect trafficking monocytes from blood to brain as it occurs in human disease. Fourth, receptor and coreceptor restrictions for viral infection can by bypassed by using pseudotyped virus. The model uses syngeneic murine BMM infected with a HIV-1/VSV-pseudotyped virus to bypass such restrictions for HIV-1 infection. In HIV/VSV HIVE mice, infected mouse macrophages implanted in the brain were eliminated faster when compared with SCID mouse model (72). This may be due to an intact immune surveillance system resulting in more limited neurodegeneration around the lesion observed. HIV/VSV infection of BMM is a one-step infection of mouse cells with no secondary infection. When HIV/VSV-infected BMM are injected into the mouse brain, HIV proteins are expressed by the infected BMM; however, the infection cannot spread to other mouse cells, including either uninfected remaining BMM or microglia, because the progeny virus from infected BMM is HIV, but not HIV/VSV. This model also lacks the formation of HIV-infected multinucleated giant cells in the brain that is the characteristic of the SCID mouse model used in previous studies. Nonetheless, in the new model, HIV-induced neuropathology was significant in enabling the analyses of anti-inflammatory responses and neuroprotection. Astroglial responses were more diffuse than microglial activation. The model provides the abilities to study the means to affect neural repair or neuroprotection at earlier stages of viral infection and at times when the brain is most likely to be positively affected by immune-restorative therapies.

By using this model of HIVE, we now demonstrate that therapeutic immunization with COP-1 can positively affect disease outcomes independent of antiretroviral activities. This is manifested, in part, by modulating microglia and astrocyte pro- and anti-inflammatory cytokine responses and iNOS. Interestingly, such COP-1-specific immune responses did not affect antiretroviral T cell activities. Significant neuroprotective responses followed neurotrophic factor production with T cell infiltration. Moreover, positive outcomes for neuroprogenitor cell formation provide further support of the multifaceted role that COP-1 can play in neuroprotective responses (15). Nonetheless, based on the rapid disease progression and neuronal degenerative responses seen in the current model, administration of COP-1 after the development of HIVE was not feasible. Indeed, the animals develop encephalitis after injection of HIV-1-infected BMM within 3–5 days, and 7 days are required to generate a robust T cell response. Thus, given these limitations, in attempts to maximize T cell neuroprotective responses, we followed previously published COP-1 immunization regimens (73). Thus, our observations suggest that modulation of

T cell function could affect neuroprotective responses during disease. Because infected brain mononuclear phagocytes produce chemotactic cytokines that recruit inflammatory cells to the site of inflammation (3), influx of Th2 T cells with anti-inflammatory phenotype may change the microglial phenotype to support neuronal survival and renewal.

In the present study, COP-1-induced T cell responses led to the generation of a Th2 anti-inflammatory phenotype with attenuation of neuroinflammation and neuroprotective responses. COP-1, a Food and Drug Administration-approved drug for the treatment of multiple sclerosis (74), shows potential for its use in several other neurodegenerative diseases. Our results extend these findings and demonstrate that COP-1-modulated immune responses attenuate inflammatory activities in the brain during active neurodegenerative processes and lead to neuroprotective outcomes. Such findings support a more generalized efficacy of COP-1 immunization in divergent models of human neurologic disorders, including spinal cord injury, glaucoma, amyotrophic lateral sclerosis, AD, and PD (18, 23, 75, 76).

Our results also demonstrate that immunization results in COP-1-reactive lymphocytes secreting IL-4 and IL-10 with reduced IL-12p70. COP-1-reactive T cells were tested for proliferative responses in vitro with an index of 2.0 compared with unstimulated controls in [^{3}H]thymidine incorporation assays (data not shown). Increased expression of IL-4 was observed in the brain tissue. COP-1-reactive T cells were previously shown to secrete IL-4, IL-5, IL-6, IL-10, and TGF-β (25, 77, 78). Generated in the periphery, COP-1-specific regulatory T cells readily cross the blood brain barrier and exert therapeutic effects for damaged neural tissue (22, 79, 80), as well as suppress the proliferation of MBP-specific T cells (81, 82). Lymphocytes present in the lesion area, determined by CD3 expression, paralleled the degree of inflammation. COP-1-induced peripheral anti-inflammatory responses may contribute to diminished ingress of lymphocytes into the brain as activated T cells preferentially enter the CNS after lentiviral infections (58). Foxp3 expression in COP-1-immunized animals suggests the presence of a regulatory T cell pool (83) in the lymphocyte population around the injected area. The increase in regulatory T cells in COP-1-treated animals demonstrates the importance of specific T cell subsets in neuroprotective and anti-inflammatory activities in neurologic and neuropsychiatric disorders (84, 85). BDNF expression was significantly increased in HIVE animals after COP-1 treatment around the lesion areas. COP-1 treatment modulates neurotrophin expression and is linked to neuroprotection. Putative mechanisms involved in the regulation of inflammation and neuroprotection are illustrated schematically in Fig. 10.

Adoptive transfer of COP-1-immunized T cells into SCID mice injected with HIV/VSV BMM significantly reduced neuroinflammation in HIVE animals and afforded neuroprotection at levels comparable to those obtained by active immunization (data not shown). These data provided additional confirmation to the specificity of adaptive immune-mediated neuroprotection in the HIVE animal system. Moreover, the data further demonstrate that COP-1-induced neuroprotection is mediated through T cell responses. Nonetheless, this is most likely not the only mechanism. Indeed, we recently showed that COP-1 can also induce T cell-independent neuroprotective activities for HIVE (Gorantla, S., J. Liu, T. Wang, A. Holguin, H. M. Sneller, H. Dou, J. Kipnis, L. Poluektova, and H. E. Gendelman, unpublished observations). In the present study, we also used two different immunization regimens with or without the adjuvant to open up the possible implications for human use. The presence of subtle, but significant protection of hippocampal neurogenesis in COP-1 treatment suggests the ability of anti-inflammatory T cell activities to support neuroregeneration; however, whether these are direct T cell mediated (86) has yet to be determined.

Our results, taken together, provide a rationale for the use of immunization strategies independent of those that induce antiretroviral responses for therapeutic use in HIV-1-associated cognitive impairments. A strategy, such as the one described in this study, that promotes activation of T regulatory cells accelerates anti-inflammatory responses to the sites of active disease. This, coupled with a significant local production of neurotrophic factors in a safe and easily administered manner, has clear potential for human use. The broad-based potential efficacy of this approach for many neurodegenerative disorders, in which inflammation plays a central role in disease pathogenesis, makes these observations appealing toward the development of broader based therapies for human disease.

Acknowledgments

We are grateful to Dr. Lee Mosley of University of Nebraska Medical Center and Dr. Mary Jane Potash of Columbia University for critical reading of this manuscript and their advice. We also thank Robin Taylor of the University of Nebraska Medical Center for administrative assistance. We also acknowledge the following sources for providing the reagents: National Institutes of Health AIDS reagent program for pYU-2, Dr. B. Cullen, Duke University for pHIT/G, and Dr. T. Seki at Juntendo University for anti-PSA-NCAM Ab.

Disclosures

The authors have no financial conflict of interest.

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