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Modulation of Innate Immunity by Copolymer-1 Leads to Neuroprotection in Murine HIV-1 Encephalitis

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Abstract

Virus-infected and immune competent mononuclear phagocytes (MP; perivascular macrophages and microglia) drive the neuropathogenesis of human immunodeficiency virus type one (HIV-1) infection. Modulation of the MP phenotype from neurodestructive to neuroprotective underlies adjunctive therapeutic strategies for human disease. We reasoned that, as Copolymer-1 (Cop-1) can induce neuroprotective activities in a number of neuroinflammatory and neurodegenerative disorders, it could directly modulate HIV-1 infected MP neurotoxic activities. We now demonstrate that, in laboratory assays, Cop-1-stimulated virus-infected human monocyte-derived macrophages protect against neuronal injury and elicit anti-retroviral activities. Severe combined immune deficient (SCID) mice were stereotactically injected with HIV-1 infected human monocyte-derived macrophages, into the basal ganglia, to induce HIV-1 encephalitis (HIVE). Cop-1 was administered subcutaneously for 7 days. In HIVE mice Cop-1 treatment led to anti-inflammatory and neuroprotective responses. Reduced micro- and astro- gliosis, and conserved NeuN/MAP-2 levels were observed in virus affected brain regions in Cop-1-treated mice. These were linked to interleukin-10 and brain-derived neurotrophic factor expression and downregulation of inducible nitric oxide synthase. The data, taken together, demonstrate that Cop-1 can modulate innate immunity and, as such, improve disease outcomes in an animal model of HIVE.

Keywords

Copolymer-1; neuroprotection; microglia; anti-inflammatory; human immunodeficiency virus; severe combined immunodeficient mice; HIV encephalitis

Introduction

Neurological complications of HIV-1 infection remain a common cause of morbidity even after widespread anti-retroviral therapies (ART). HIV resistance to ART, drug compliance, limited pharmacokinetics, toxic side effects and an inability to clear viral reservoirs, can, in part, explain disease progression. Such events remain associated with cognitive, behavioral, and motor disturbances in infected people (McArthur 2004; Navia and Rostasy 2005). For

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HIV-1 associated cognitive impairments, considerable evidence shows that impaired innate macrophage and microglial activities affect viral infection and mental deterioration (Gray et al. 2001; Kalams and Walker 1995; Navia and Rostasy 2005; Sopper et al. 1998). Indeed, the major factors involved in inducing neural injury in HIV-1-associated dementia (HAD) are viral and cellular products secreted by immune competent mononuclear phagocytes (MP; perivascular macrophages and microglia) (Aksenov et al. 2003; Garden 2002; Gendelman et al. 2004; Kanmogne et al. 2002). These include, but are not limited to, the HIV-1 proteins like gp120 and tat, pro-inflammatory cytokines, arachidonic acid and its metabolites, quinolinic acid, and glutamate (Kaul and Lipton 2005; Rostasy et al. 2005; Smith et al. 2001). If modulation of MP functions and the resultant inflammation can be achieved during disease, the tempo and progression of HIV-1-associated cognitive impairment could be significantly slowed (Kadiu et al. 2005). This is the principal focus for adjunctive therapies (Dou et al. 2004).

Copolymer-1 (Cop-1, Glatiramer acetate, Copaxone®), an FDA approved immunomodulatory drug currently used for the treatment of Multiple Sclerosis (MS) (Arnon and Aharoni 2004), can promote Th2 regulatory T lymphocyte responses affecting the production of anti-inflammatory cytokines in mice and humans (Aharoni et al. 1997; Miller et al. 1998). As Cop-1-specific T cells are known to modulate microglial and astrocyte phenotypes and slow neurodegeneration (Aharoni et al. 2000; Frenkel et al. 2003; Kipnis et al. 2000) we reasoned that it could also be used as an adjunctive therapy for NeuroAIDS. Indeed, Cop-1 immunization can generate an anti-inflammatory microenvironment and increase the production of neurotrophins at the site of neural injury (Aharoni et al. 2003; Benner et al. 2004). Apropos of treatment, Cop-1 is an effective immune modulator inducing neuronal protection in animal models of experimental autoimmune encephalomyelitis, optic nerve crush, Parkinson's and Alzheimer's disease (Arnon and Sela 2003; Benner et al. 2004; Frenkel et al. 2005; Kipnis et al. 2000). The mechanisms of action for Cop-1 are incompletely understood but, in part, include its direct engagement of dendritic cells and neurons (Kipnis, J. personal communication). Cop-1 can block monocyte activation, affect the release of tumor necrosis factor alpha (TNF- α) and interleukin-12 (IL-12) (Kim et al. 2004). Cop-1 can also inhibit dendritic cells to produce inflammatory mediators, as well as, impair IL-12p70 secretion in response to lipopolysaccharide (Jung et al. 2004; Losy et al. 2002; Vieira et al. 2003).

In the current report, we show that Cop-1 directly modulates innate immunity and reduces inflammatory responses that improve neuropathological outcomes in a rodent model of HIV-1 encephalitis (HIVE). Cop-1 led to neuroprotection against HIVE with reduced expression of inducible nitric oxide synthase (iNOS), and increased IL-10 and brain derived neurotrophic factor (BDNF). These observations provide, a proof-of concept, for the use of Cop-1 as an adjunctive therapy for HIV-1-associated cognitive impairments.

Materials and Methods

Isolation of human monocytes, culture and collection of conditioned media

Human peripheral blood mononuclear cells were isolated from leukopaks of HIV-1 and 2, and hepatitis B virus seronegative donors. Monocytes were purified by counter-current centrifugal elutriation and were cultured in complete medium consisting of DMEM supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine, gentamicin (50 μ g/ml), ciprofloxacin (10 μ g/ml), and MCSF (a generous gift from Wyeth Inc., Cambridge, MA) used at 1000 U/ml. All cell culture reagents excluding MCSF, were procured from Invitrogen, Carlsbad, CA. Monocytes were cultured for 7 days to differentiate them into monocyte-derived macrophages (MDM). Half media exchanges were done with fresh complete media once in every 2-3 days. At day 7, MDM cultivated in 24 well culture plates at a density of 1.0×10^6 cells/well were infected with HIV-1_{ADA} at an MOI of 0.01 (Gendelman et al. 1988). HIV-1 infected MDM

conditioned media (MCM) was collected on day 5 after viral infection. For HIV-1 and Cop-1 treated MCM, MDM were treated with 25 or 50 µg/ml Cop-1 (Sigma-Aldrich, St Louis, MO) at 24 h before HIV-1 infection, and the supernatants were collected on day 5 after viral infection. MCM from Cop-1 only treated cells were collected from parallel cultures. Replicate experiments were performed with clinical grade Cop-1 (TEVA Neuroscience, Kansas City, MO). One day before collecting MCM, Cop-1 was removed from all cultures by replacing with complete media without Cop-1. MCM was collected and clarified by centrifuging at 600×g and stored at -80°C until use for neuroprotection assays (see below).

Isolation and culture of mouse fetal neurons

Embryonic day 18 old pups were harvested from anesthetized pregnant dams of C57/B6 strain inbred in our animal facility at University of Nebraska Medical Center. Animals were maintained in sterile microisolator cages under pathogen-free conditions in accordance with ethical guidelines for care of laboratory animals at the University of Nebraska Medical Center set forth by the National Institutes of Health. Cerebral cortices were isolated and digested using 0.25% trypsin (Invitrogen, Carlsbad, CA). Cells were seeded at a density of 0.12×10^6 /well on poly-D-lysine coated coverslips placed in 24 well plates and maintained in neurobasal medium supplemented with B27, penicillin/streptomycin and 5mM L-glutamine (Invitrogen). The purity of neural cells was determined by staining with microtubule-associated protein-2 (MAP-2, a mature neuronal marker) antibody. More than 80% of MAP-2 positive cells were obtained in neuronal cultures after 2 weeks. MCM obtained from Cop-1, HIV-1 or HIV-1/Cop-1 treated MDM were added to the neuronal cultures to test neurotoxicity. Neuronal apoptosis was measured after 24 h.

Neuronal apoptosis

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining was performed using the *in situ* cell death detection kit, *AP* (Roche applied science, Indianapolis, IN) according to the manufacturer's instructions. Apoptotic cells were identified with TUNEL under fluorescent microscope, and were normalized to 4',6'-diamidino-2-phenylindole (DAPI) nuclei staining. TUNEL positive cells were scored and the percent apoptotic neurons were calculated to total DAPI-positive cells.

Immunocytochemistry

Neuronal cultures after being exposed to HIV-MCM treated with or without Cop-1 for 24 h were immunocytochemically stained for MAP-2 and NeuN antigens. Antibodies were obtained from Chemicon International, Temecula, CA. Alexafluor conjugated secondary antibodies (Molecular Probes, Eugene, OR) were used and laser scanning images were obtained using a Nikon Swept-field laser confocal microscope (Nikon Instruments, New York, NY).

Reverse transcriptase (RT) and cell viability assays

MDM were infected with HIV-1_{ADA} as described above with or without Cop-1 (25 and 50 µg/ml) added 24 h before infection. The supernatants were collected at different time points until day 14, to estimate HIV-1 replication by measuring RT activity. RT activity was determined by incubating 10 µl of sample with a reaction mixture consisting of 0.05% Nonidet P-40 (Sigma) and [³H]dTTP (2Ci/mmol; Amersham Corp., Arlington Heights, IL) in Tris-HCl buffer (pH 7.9) for 24 h at 37°C. Radiolabeled nucleotides were precipitated on paper filters in an automatic cell harvester (Skatron, Sterling, VA) and incorporated activity was measured by liquid scintillation spectroscopy. Cop-1 added directly to viral preparations served as controls to detect whether Cop-1 affects HIV RT activity. Cell viability was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The assay is based on the ability of active mitochondrial dehydrogenase to convert dissolved MTT to water-

insoluble purple formazan crystals. Supernatants, from MDM cultures with or without Cop-1 and HIV-1 infection, were completely aspirated and 5 mg/ml solution of MTT in complete medium was added to each well and incubated at 37°C for 1 h. After the incubation MTT solution was removed and 100% Dimethyl sulfoxide was added to each well. The color developed was read at 490nm using a spectrophotometric plate reader.

HIVE mice

Four-week old male C.B-17/SCID mice were purchased (Charles River Laboratories) and maintained in sterile microisolator cages under pathogen-free conditions in accordance with the ethical guidelines for care of laboratory animals at the University of Nebraska Medical Center and the National Institutes of Health. Monocytes were cultured in suspension in a teflon flask for 7 d to generate MDM, and they were infected with HIV-1_{ADA} (a macrophage tropic strain) at a multiplicity of infection (MOI) of 0.01 for 24 h. To induce HIVE in mice, HIV-1_{ADA}-infected MDM (3×10^5 cells in 5 μ l) were injected intracerebrally (*i.c.*) into the basal ganglia stereotactically with previously described co-ordinates (Persidsky et al. 1996). Cop-1 was administered subcutaneously (*s.c.*) at 75 μ g/mouse/day beginning from the day of HIVE induction until day 7, the day of sacrifice when inflammation and neuronal injury are at peak levels (Persidsky et al. 1996). Control mice were left untreated ($n=12-14$ per group per experiment, 6-7 for immunohistology and 6-7 for RT-PCR). Sham injected (5 μ l saline) mice were also included to serve as controls. A total of three separate experiments were performed with the same number of animals.

Immunohistochemical assays

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- μ m-thick) sections were cut from the injection site to the hippocampus. Sections were deparaffinized and immunohistochemical staining followed a basic indirect-immunostaining protocol, using antigen retrieval by heating to 95°C in 0.01 M citrate buffer for 30 min. Murine microglia and astrocytes were detected with rabbit polyclonal antibodies to ionizing calcium-binding adaptor molecule 1 (Iba-1) (1:500, Wako, Richmond, VA) and glial fibrillary acidic protein (GFAP) (1:1000, Dako, Carpinteria, CA) respectively. Antibodies to Neuronal Nuclei (NeuN) (1/100), MAP-2 (1/1000; Chemicon) and phosphorylated form of heavy neurofilament subunit (phos-NF, clone 2F11 at 1/100, Dako) were used to identify neurons. Antibodies to human CD68 (clone KP1 at 1/100) and HIV-1 p24 were obtained from Dako. The polymer-based horseradish peroxidase conjugated anti-mouse and anti-rabbit Dako EnVision systems and polymer-based alkaline phosphatase conjugated PowerVision TM (ImmunoVision Technologies Co., Daly City, CA) systems were used as secondary detection reagents, and 3,3'-diaminobenzidine (DAB, Dako) was used as the chromogen. For double staining, primary antibodies were applied simultaneously and development steps performed separately. All paraffin-embedded sections were counterstained with Mayer's hematoxylin. Deletion of primary antibodies served as controls. Images were obtained by Optronics digital camera (Buffalo Grove, IL) fixed to Nikon Eclipse E800 (Nikon Instruments Inc., Melville, NY) using MagnaFire 2.0 software (Goleta, CA). Quantification of immunostaining was performed using Image-Pro Plus (Version 4.0, Media Cybernetics, Silver Spring, MD) on serial coronal brain sections.

Real-time RT-PCR

Total RNA from brain sections (2 mm thick) containing the injection line, was extracted using TRIzol method (Invitrogen, Carlsbad, CA). RNA was cleaned on RNA mini columns (Qiagen, Valencia, CA) with DNase treatment, and reverse transcribed with random hexamers and MMLV reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed with

cDNA using an ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA). Murine-specific primer pairs were: GFAP, 5'-ACTGGGTACCATGCCACGTT-3' and 5'-GGGAGTGGAGGAGTCATTCG-3'; Mac-1, 5'-GCCAATGCAACAGGTGCATAT-3' and 5'-CACACATCGGTGGCTGGTAG-3'; IL-10, 5'-CAGTTATTGTCTTCCCGGCTGTA-3' and 5'-CTATGCTGCCTGCTCTTACTGACT-3'; iNOS, 5'-GGCAGCCTGTGAGACCTTTG-3' and 5'-GAAGCGTTTCGGGATCTGAA-3'; and NGF, 5'-TTCCATCCAGTTGCCTTCTTG-3' and 5'-GAAGGCCGTGGTTGTCACC-3'. A SYBR Green I detection system was employed, and quantification was done using standard curve method as described in user bulletin #2 obtained with ABI PRISM 7000 sequence detector. BDNF expression was analyzed using TaqMan gene expression assays designed for mouse. All PCR reagents were obtained from Applied Biosystems. Gene expression was normalized to GAPDH and was used as an endogenous control.

Statistical analysis

The results were expressed as mean $\pm$ SEM for each group with 6-7 animals. Statistical significance between groups was analyzed by Student's *t*-test and one-way analysis of variance (ANOVA) using Microsoft Excel. Differences were considered statistically significant at $P < 0.05$.

Results

Cop-1 protects neurons from HIV-1 infected MDM toxicity

We utilized laboratory assays that included primary cell cultures of mouse fetal neurons to determine whether Cop-1 can modulate HIV-1 infected MDM secretory activities leading to neuroprotective outcomes. Conditioned media was collected from MDM treated with Cop-1 and/or infected with HIV-1. Neuronal cultures were exposed to the MDM conditioned media, and the neuronal integrity was determined by staining immunocytochemically for MAP-2 and NeuN (Figure 1A). Numbers of apoptotic neurons were determined and the neuronal apoptosis was quantified by combined TUNEL/DAPI staining. These results are illustrated in Figure 1B. MCM from HIV-infected MDM showed significant neurotoxicity with respect to MAP-2 loss and also increased number of apoptotic neurons compared to control MCM. Conditioned media collected from Cop-1-treated HIV-infected MDM significantly protected neurons demonstrated by MAP-2 staining and reduced apoptosis. MCM collected from both 25 and 50 $\mu\text{g/ml}$ of Cop-1 showed significant neuronal protection by MAP-2 stained dendrites. Reduction in apoptotic cells was from $21.36 \pm 3.2\%$ to $10.96 \pm 1.1\%$ (HIV-MCM vs HIV/Cop-1-MCM, $P < 0.01$). Neuroprotection was significant at both 25 and 50 $\mu\text{g/ml}$ concentrations (Figure 1B). The experiment was performed three separate times in triplicate determinations. Neuroprotection was also determined on human neurons and the supernatants from Cop-1 treated HIV-infected MDM showed significant reduction in neuronal apoptosis (data not shown).

Cop-1 restricts HIV-1 replication in MDM

In order to determine the potential mechanisms for Cop-1-induced neuronal protection for examined its effects on HIV-1 replication in MDM. When MDM were treated with Cop-1 at 24 h before infection, progeny virion production was significantly reduced until day 10 in presence of 50 $\mu\text{g/ml}$ Cop-1 when compared to untreated HIV-1 infected MDM controls (Figure 2A). Nonetheless, by 12 days after infection levels of viral replication in Cop-1-treated and control virus-infected MDM were similar. In presence of Cop-1, the number of multinucleated giant cells, a hallmark of HIV-1 infection, was significantly reduced when compared to HIV-1 infected macrophages without treatment. Cell viability analyzed by MTT assay demonstrated that Cop-1 used at 50 $\mu\text{g/ml}$ did not affect MDM viability (Figure 2B).

Murine HIVE

To extend our *in vitro* studies of Cop-1 induced neuroprotection, we used a murine model of HIVE. Human MDM from normal donors were infected with HIV-1 and injected into the brains of SCID mice to induce cognitive, neuropathologic, neuroimmune, and physiologic abnormalities reflecting the disease of infected human host (Persidsky et al. 1996; Tyor et al. 1993; Zink et al. 2002). The generation of this model and the resulting neuropathological outcomes are illustrated in Figure 3. SCID mice are devoid of a functional adaptive immune system but have an intact innate immunity. This rodent system permits the study of innate immune responses generated from macrophage secretions. *First*, since HIV-1 infected people develop cognitive impairment coordinated with significant immune suppression (McArthur et al. 2005), if Cop-1 may be used to treat human disease, it must elicit an effect independent of T cells. *Second*, prior studies showed immunomodulatory effects of Cop-1 on innate immunity including macrophages and dendritic cells (Jung et al. 2004; Vieira et al. 2003). Moreover, recent studies conducted in our laboratories demonstrated T-cell independent neuroprotective mechanisms of Cop-1, both through dendritic cells and neurons (Kipnis et al. manuscript submitted). Histopathological effects observed in brain tissue of immune deficient HIVE mice showed typical multinucleated giant cell encephalitis, identified by antibody to human CD68 (Figure 3). In SCID mice Cop-1 was administered for 7 days from the day of HIVE induction until sacrifice.

Cop-1 modulates neuroinflammatory responses in HIVE mice

Immunopathology of brain sections revealed that the number of human MDM, as identified by human CD68 staining, and the amount of HIV infection, analyzed by HIV-1p24 staining was not affected by Cop-1 administration when compared to controls, 24.6 ± 6.1 vs 23.1 ± 5.4 and 12.3 ± 2.7 vs 11.7 ± 5.0 (Figure 4). Stained cells per section were quantified as percent stained area of the total field of view, and the average was determined from three sections per mouse and six mice per group. Morphological changes in astrocytes were detected by immunostaining for GFAP expression, and microglial activation was identified by staining for expression of Iba-1. We quantified GFAP-positive astrocytes and Iba-1 reactive microglia on serial coronal sections. Figure 4 shows GFAP and Iba-1 staining, where both astrogliosis and microglial activation was reduced in Cop-1 immunized mice, but did not reach significance ($P = 0.058$ and 0.09 , respectively).

In order to substantiate the immunohistochemical data, we used real time RT-PCR assays for affected brain tissue. Quantification of GFAP (astrogliosis) and Mac-1 (microglial activation) gene expression in ipsilateral (Ips) and contralateral (Con) brain tissue sites are shown in Figure 5. In the ipsilateral sites, GFAP and Mac-1 gene expression was reduced by Cop-1 immunization but did not reach significance compared to controls ($P = 0.07$ and 0.09 , respectively). However, parallel studies demonstrated a significant decrease in iNOS ($P < 0.05$) with higher levels of IL-10 ($P < 0.05$) compared to non-immunized controls. RT-PCR analysis for neurotrophins demonstrated a 30% increase in BDNF ($P < 0.05$) and 25% increase in nerve growth factor (NGF, $P < 0.05$) in the ipsilateral hemispheres from Cop-1 treated animals, supporting the neuroprotection in these animals. IL-10 ($P < 0.01$), BDNF ($P < 0.05$) and NGF ($P < 0.05$) levels were also increased in the contralateral hemispheres in Cop-1 treated animals. RT-PCR analysis from sham injected brains showed IL-10, BDNF and NGF levels are equivalent to the levels observed in unmanipulated animals (data not shown). The modest increases in cytokine levels in the contralateral hemisphere likely reflects the fact that MDM migrate amongst hemispheres or alternatively, that soluble proteins diffuse within the brain itself. Such results were previously shown by our group in this HIVE model (Persidsky et al. 1997).

Cop-1-induced neuroprotection in HIVE mice

Cop-1 effects on neuronal damage were studied by immunostaining the brain sections with antibodies to MAP-2, NeuN and phos-NF. In sham injected brains, reduction in MAP-2 staining was seen only at the injection line (data not shown). In HIVE animals, neuronal loss was extended beyond the areas of MDM (Figure 4). Neuronal protection was observed with increases in NeuN/MAP-2 positive dendrites and neurites in Cop-1 treated animals (Figure 6). Quantitative measurement of NeuN/MAP-2 stained area also revealed significant increase in immunopositive staining in Cop-1 animals with $50.5\% \pm 3.4$ positive stained area when compared to controls with only $28.6\% \pm 6.7$ ($P < 0.05$). Antibody to phos-NF was used to identify neuronal cell bodies undergoing degeneration (Dou et al. 2005). Phosphorylated heavy neurofilament subunits are normally restricted to axons, but in trauma they are shown to accumulate in neuronal perikarya which are normally devoid of phosphorylated subunit. Staining for phos-NF showed degenerating neurons around HIV-1 infected MDM areas. The number of degenerating neurons was significantly reduced with Cop-1, as shown in Figure 6 ($P < 0.01$).

Discussion

Brain MP activation and persistent viral infection drive a metabolic encephalopathy that, in its most severe form, leads to significant cognitive, motor and behavioral abnormalities and is termed HAD (Gendelman et al. 2004). HIVE is multinucleated giant cell encephalitis, characteristic of monocyte-macrophage brain infiltration and formation of microglial nodules. Such pathologic, behavioral, and physiologic parts of human disease are mirrored in SCID mice where HIV-1 infected human MDM are injected into the basal ganglia (Persidsky et al. 1996; Zink et al. 2001). SCID mice injected with human HIV-1 MDM showed significant and sustained microglial and astrocytes responses. A strong linkage exists between neuroinflammation and neurodegenerative processes not only in HIVE but also in many other neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Alzheimer's and Parkinson's diseases (reviewed by (Kadiu et al. 2005).

Cop-1 has been chosen since it has a translational potential for the treatment of human disease for several reasons. *First*, Cop-1 a synthetic amino acid copolymer, is currently an approved drug for the treatment of MS (Adorini 2004; Wolinsky 2004). *Second*, Cop-1 was shown to block Th1 cytokines such as interferon gamma that can impede neurogenesis which are reversed by IL-4 resulting in attenuated pro-inflammatory (such as TNF) production, and induction of IGF-I (Butovsky et al. 2006). Cop-1 modulates monocyte-macrophage phenotype (Jung et al. 2004; Kim et al. 2004; Li et al. 1998). *Third*, evidence abounds that reduction of CNS neuroinflammation, produced as a consequence of HIV-1 infection, can affect disease outcomes (Persidsky and Gendelman 2002; Rostasy 2005). *Fourth*, Cop-1 induced Th2 adaptive immune responses can affect microglial responses and lead to neuroprotection in animal models of neurodegenerative, metabolic and traumatic disorders including optic nerve injury, head trauma, PD and AD (Aharoni et al. 2000; Benner et al. 2004; Kipnis et al. 2003).

We used both *in vitro* and *in vivo* models to demonstrate that Cop-1 can modulate innate immune cells and confer neuroprotection. *In vitro* studies showed that conditioned media obtained from Cop-1 treated HIV-1 infected MDM could protect neurons significantly. The number of apoptotic neurons HIV conditioned macrophage media were reduced when the neurons were exposed to Cop-1 treated HIV infected macrophage media. The neuroprotective ability of Cop-1 in the *in vivo* model was conferred by the reduction in phosphorylated neurofilament expressing neurons and also preservation of MAP-2/NeuN positive dendrites/nuclei with a reduction in lesion size around the injection line. Cop-1 showed significant reduction and delayed establishment of productive HIV-1 replication *in vitro*. However, Cop-1 did not affect the number of MDM or HIV-1 infection in the brains of SCID mice with HIVE.

This may be due to the levels of Cop-1 *in vivo* was not enough to regulate HIV-1 infection, or may be at day 7 when the animals were sacrificed for morphological analysis, if there was any delayed infection, the levels might have reached to normal at the time of sacrifice. Although HIVE is major viral-associated inflammatory response, studies in our laboratory showed that adjunct therapy with sodium valproate or lithium provided neuroprotection without altering macrophage HIV-1 infection (Dou et al. 2003; Dou et al. 2005).

The mechanisms of action for Cop-1 are likely multifaceted. The ability of Cop-1 to directly affect pro-inflammatory responses through its direct engagement of macrophages and dendritic cells and neurons likely explain the neuroprotective innate immune responses in our SCID HIVE mice (Jung et al. 2004; Vieira et al. 2003). A recent report showed that Cop-1 treatment promotes the development of type II monocytes characterized by anti-inflammatory cytokine secretion (Weber et al. 2007). Cop-1 can also modulate RANTES levels in glia (Li and Bever 2001; Li et al. 2001) and differential expression of RANTES along with other chemokines was shown to effect HIV-1 replication (Sanders et al. 1998). Modulation of MP innate immunity could occur through a number of mechanisms. Indeed, MP secrete a plethora of bioactive molecules that can damage the brain directly. MPs are also a potent source of chemotactic cytokines that can recruit nascent leukocytes to sites of inflammation and as such perpetuate the viral reservoir and sources of neurotoxins (Sopper et al. 1998). MP can secrete other factors to restrict virus infection or replication in proximal cells, most notably interferons (Lokensgard et al. 1997; Poli et al. 1991). Cop-1 induces anti-inflammatory cytokines and as such may induce neuroprotective activities. We have in fact seen such responses during studies of T cell modulation by Cop-1 in a chronic HIVE rodent model (Gorantla et al. 2007). In addition, IL-10 expression by microglia has a broader role in neuroprotection by its ability to induce neurotrophic factor production (Aharoni et al. 2003). Induction of IL-10 by Cop-1 with its direct effects on the innate immune system seems to have major role in the neuroprotection observed in immune deficient HIVE mice. Production of pro-inflammatory cytokines (for example, TNF- α and IL-12) is reduced by IL-10 and TGF- β (Jung et al. 2004). Neuroprotection in Cop-1 treated animals was confirmed with increase in MAP-2 staining and reduced phosphorylated neurofilament positive neurons. These findings support the notion that Cop-1 was neuroprotective even without the involvement of adaptive immune responses, but possibly through direct or indirect interactions with peripheral monocyte/macrophages and/or brain microglia.

One important unresolved question is how to best translate these animals studies to clinical trials. The results shown both in the current report and in a companion paper (Gorantla et al. 2007) support Cop-1's use as an adjuvant therapy for neuroAIDS. This is true both in both immunosuppression and immune competent settings. In our own neuroAIDS programs support for bench to bedside research efforts has been realized (Schifitto et al. 2006). Moreover, the infrastructure for such a clinical trial is in place as a phase II study of Cop-1 was recently completed and fully evaluated for ALS patients (Gordon et al. 2006; Mosley et al. 2007). Thus, the clinical framework is in place to continue this work on a clinical level when deemed appropriate.

Altogether, our results provide a rationale for the use of Cop-1 as an adjunctive therapy for HIV-1-associated cognitive impairments and supports our own previous works in regards to adaptive immunity and neuroprotection in HIVE (Gorantla et al. 2007). A strategy, such as the one described in this report, that promotes anti-inflammatory activities and neurotrophin release and could affect neuroprotection in a safe and easy administered manner has clear potential for use in human disease. The broad-based potential efficacy of this approach for other neurodegenerative disorders makes such ideas appealing.

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Figure 1.

Neuroprotection mediated by Cop-1 exposed HIV-1 infected MDM. Human MDM were treated with Cop-1 at 25 or 50 μg/ml and 24 h later infected with HIV-1_{ADA} at an MOI of 0.01. Macrophage conditioned media (MCM) were collected at day 5 after infection and added to mouse cortical neuronal cultures. Neurons were stained immunocytochemically for MAP-2 and NeuN (A). Confocal images demonstrated that MAP-2 staining was reduced process formation and intensity after neuronal exposure to HIV-MCM. In contrast, dendritic processes were protected by MCM collected from Cop-1 (50 μg/ml) treated HIV-1 infected MDM. Original magnification ×400. Neuronal apoptosis was measured by combined TUNEL/DAPI staining and the images captured using fluorescent microscope are shown (B). The fluorescent

apoptotic neurons were counted, and the percentage was calculated by dividing the number of TUNEL-positive cells by total DAPI- positive cells (B). Values are expressed as mean $\pm$ SEM of triplicate cultures. The results are representative of three independent experiments performed in triplicate determinations.

Figure 2.

Inhibition of viral replication by Cop-1 in MDM. MDM were treated with 25 or 50 µg/ml Cop-1 for 24 h and then infected with HIV-1_{ADA}. Controls are infected with HIV-1 without the Cop-1 treatment. Supernatants were collected at different days after infection to determine HIV-1 replication by using the assay for RT activity of the virus (A). Cop-1 significantly delayed the establishment of HIV-1 infection with 50 µg/ml concentration. Values are mean ± SEM of quadruplicate cultures. **P* < 0.05 compared to HIV-infected controls. Viability of MDM at end point (day 14) was determined by MTT assay (B). Cop-1 did not show any toxicity on MDM at the concentrations used. Values are mean ± SEM of quadruplicate cultures. The viability of HIV infected MDM in presence of 50 µg/ml Cop-1 was significantly higher (**P* <

0.05, analyzed by Student's *t*-test) compared to HIV infected MDM. The results shown are representative of three independent experiments.

SCID-HIVE mice

Figure 3.

Schematic diagram SCID-HIVE mouse model. SCID-HIVE mouse model used to study the regulation of innate immune system in the absence of functional T and B lymphocytes. Human monocytes obtained after elutriation were differentiated into MDM, and HIV-1_{ADA} infected human MDM are injected into the basal ganglia of mouse brain. Cop-1 was administered at 75 µg/mouse in 100 µl saline, *s.c.*, for 7 days from the time when HIV infected MDM were injected. Animals were then sacrificed on day 7. Brain areas marked with rectangles (red is injected hemisphere and black is the uninjected side) were used for immunohistological and real time RT-PCR analyses. Immunohistology for human macrophages (CD68) shows HIV-1 infected multinucleated giant cells (marked with arrow heads).

Figure 4.

HIV-1 infection and brain inflammation in SCID-HIVE mice. Immunohistological images of the brain region (basal ganglia) injected with HIV-1-infected MDM in control and Cop-1 treated mice are illustrated in the figure. Serial 5 μ m sections containing injection area with HIV-1 infected MDM were stained for human CD68 (A, B), HIV-1 p24 (C, D), GFAP (E, F) and Iba-1 (G, H). No differences were detected in numbers of human cells (CD68 staining) and HIV-1 p24 positive cells. Reduction in astrogliosis (GFAP) and microglial activation (Iba-1) was not substantial with Cop-1 treatment. Original magnification, 100 $\times$. Quantification by digital image analysis, as represented in bar graphs, also reflected that the decrease in GFAP and Iba-1 staining was not significant in Cop-1 treated animals (black bar) when compared to

non-treated SCID-HIVE controls (open bar), # represents $P < 0.1$ as analyzed using Student's t -test. Values are expressed as mean $\pm$ SEM from 6 animals per group.

Figure 5.

Real time RT-PCR for inflammation markers, cytokines and neurotrophins in SCID-HIVE. RNA was extracted from 2mm brain section containing the ipsilateral (Ips) injection site, and the expression of GFAP, Mac-1, iNOS and IL-10 were determined by real-time RT-PCR. Corresponding sites in contralateral (Con) hemispheres were included in the analysis for comparison. GFAP and Mac-1 expression were reduced with Cop-1 treatment, but did not reach significant levels, # $P < 0.1$. However, expression of the anti-inflammatory cytokine, IL-10 was increased in both hemispheres with reduction in iNOS. Expression of the neurotrophins, BDNF and NGF, were also found to be significantly increased in Cop-1 treated brain tissue. Values are expressed as mean $\pm$ SEM from 6 animals per group. Values from Cop-1 treated HIVE animals (closed bars) are compared to corresponding hemisphere of HIVE animals (open bars), * $P < 0.05$ by Student's *t*-test and ANOVA.

Figure 6.

Neuroprotection was analyzed by staining serial sections for NeuN (brown) and MAP-2 (purple) (A, B), where lesser neuronal damage around the injection line was observed in Cop-1 treated animals. Area of lesion is outlined. Number of phos-NF positive dying neurons (C, D) around the injected area was significantly lower in Cop-1 treated animals. Original magnification for phos-NF is 400 $\times$, and 100 $\times$ for NeuN/MAP-2. The graphs represent quantification by digital image analysis in Cop-1 treated animals (black bar) compared to non-treated SCID-HIVE controls (open bar). Neuronal loss and the number of phos-NF positive dying neurons were significantly reduced in Cop-1 treated SCID-HIVE mice, $*P < 0.05$ and $**P < 0.01$ as analyzed using Student's *t*-test. Values are expressed as mean $\pm$ SEM from 6 animals per group.