Lentivirus Infection Causes Neuroinflammation and Neuronal Injury in Dorsal Root Ganglia: Pathogenic Effects of STAT-1 and Inducible Nitric Oxide Synthase

Yu Zhu, Gareth Jones, Shigeki Tsutsui, Wycliffe Opii, Shuhong Liu, Claudia Silva, D. Allan Butterfield and Christopher Power

*J Immunol* 2005; 175:1118-1126; doi: 10.4049/jimmunol.175.2.1118

http://www.jimmunol.org/content/175/2/1118

**References**

This article cites 73 articles, 26 of which you can access for free at:

http://www.jimmunol.org/content/175/2/1118.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Lentivirus Infection Causes Neuroinflammation and Neuronal Injury in Dorsal Root Ganglia: Pathogenic Effects of STAT-1 and Inducible Nitric Oxide Synthase

Yu Zhu,* Gareth Jones,* Shigeki Tsutsui,* Wycliffe Opip,† Shuhong Liu,* Claudia Silva,*, D. Allan Butterfield,† and Christopher Power‡

Distal sensory polyneuropathy (DSP) is currently the most common neurological complication of HIV infection in the developed world and is characterized by sensory neuronal injury accompanied by inflammation, which is clinically manifested as disabling pain and gait instability. We previously showed that feline immunodeficiency virus (FIV) infection of cats caused DSP together with immunosuppression in cats, similar to that observed in HIV-infected humans. In this study, we investigated the pathogenic mechanisms underlying the development of FIV-induced DSP using feline dorsal root ganglia (DRG) cultures, consisting of neurons, Schwann cells, and macrophages. FIV-infected cultures exhibited viral Ags (p24 and envelope) in macrophages accompanied by neuronal injury, indicated by neurite retraction, neuronal loss and decreased soma size, compared with mock-infected (control) cultures. FIV infection up-regulated inducible NO synthase (iNOS), STAT-1, and TNF-α mRNA levels in DRG cultures. Increased STAT-1 and iNOS mRNA levels were also observed in DRGs from FIV-infected animals relative to mock-infected controls. Similarly, immunolabeling studies of DRGs from FIV-infected animals showed that macrophages were the principal sources of STAT-1 and iNOS protein production. The iNOS inhibitor aminoguanidine reduced nitrotyrosine and protein carbonyl levels, together with preventing neuronal injury in FIV-infected DRG cultures. The present studies indicate that FIV infection of DRGs directly contributes to axonal and neuronal injury through a mechanism involving macrophage immune activation, which is mediated by STAT-1 and iNOS activation.

penicillin, 100 μg/ml prevented neuronal injury. Together with comparative analyses of in vivo features of DRG ropathogenesis of DSP focusing on FIV infection of the DRG, this study, we developed an ex vivo model for studying the neuropathogenesis of DSP focusing on FIV infection of the DRG, together with comparative analyses of in vivo features of DRG injury. The initial studies led us to hypothesize that NO might be involved in DRG injury, thereby contributing to the development of DSP. Our findings suggest that both STAT-1 and iNOS are up-regulated in DRGs during lentivirus infection and iNOS inhibition prevented neuronal injury.

Materials and Methods

Feline DRG cultures

Culture plates and chamber slides (Nunc) were coated with a 1/2 dilution (in media v/v) of matrigel (BD Biosciences). DRG from FIV seronegative healthy adult cats (12) were removed under a dissecting microscope. Cleared dissected DRGs were incubated at 37°C for 90 min in digestion media containing 0.5 mg/ml trypsin (Life Technologies), 1 mg/ml collagenase type IA (Sigma-Aldrich), and 0.1 mg/ml DNase type I (Roche Diagnostics) in DMEM (Sigma-Aldrich). Digestion media was removed by centrifuge at 1500 rpm for 5 min and cells were washed twice with culture medium (DMEM containing 10% heat-inactivated FBS, 5% horse serum (Life Technologies), 2 mM l-glutamine and 1% N-2 supplement (Life Technologies), 0.1 mg/ml penicillin/streptomycin (Life Technologies), and 5% L929 cell-conditioned medium (as a source of macrophage CSF-1). The tissue solution was triturated using a sterile glass pipette until a homogeneous cell suspension was obtained and adjusted to a concentration of 0.5 × 10^6 cells/ml. Cells were seeded into a 24-well plate (1 ml/well) or 8-well chamber slides (250 μl/well) and incubated at 37°C, 5% CO₂. On the next day, medium was changed, and every third day thereafter.

Feline macrophage cultures

Feline macrophages were isolated from the pelvic and femoral bone marrow of healthy specific pathogen-free cats, as described previously (41). The cells were cultured in DMEM containing 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated FBS, and 10% L929 cell-derived conditioned medium as a source of macrophage CSF-1 in 10% CO₂ in plastic dishes permitting cellular differentiation, resulting in monolayer cultures that were >95% pure macrophages.

Virion preparation and infection

The FIV strain used in this study was the infectious neurovirulent recombinant molecular clone, V1-Ch, derived by transfection of CrFK cells and amplification in feline PBMCs, as described previously (34). Culture supernatants from FIV-infected feline PBMC, which served as sources of infectious virus for these experiments, were cleared of cellular debris by centrifugation and filtered by limiting dilution, as described previously (18). Following 7 days of in vitro differentiation, DRG cultures were infected with FIV V1-Ch (TCID₅₀ 10²/ml; 10 μl/well for 8-well chamber slides and 100 μl/well for 24-well plates), incubated at 37°C with 5% CO₂ for 6 h, following which the cultures were washed to remove input virus, cultured under humid conditions at 37°C with 10% CO₂ in culture medium until cells were harvested at different time points (days 1 and 2 postinfection). As controls, DRG and macrophage cultures were infected with supernatant from activated uninfected (mock) PBMCs. FIV-infected and control DRG cultures were treated with 0.5 mM aminoguanidine (AG) and 5.0 μM l-Nω-(1-iminoethyl)lysine (l-NIL) (Sigma-Aldrich), following infection for 4 days.

Experimental animals and tissue collection

Specific pathogen-free neonatal kittens (day 1) were inoculated intracranially with 0.2 ml of infectious or heat-inactivated virus (control cats) in accordance with Calgary Animal Care Committee guidelines, as described previously (18). Kittens were weaned at 6 wk and monitored until 12 wk of age at which time, all animals were euthanized as previously described (18). L4 DRGs were collected and fixed in 4% PBS-buffered parafomaldehyde for 12 h at 4°C and submerged in PBS containing 20% sucrose overnight at 4°C following washing in PBS. The fixed DRGs were embedded in OTC compound, fast frozen in isopentane, and stored at −80°C until sectioning. Collected L5 DRGs were immediately frozen on dry ice and kept in −80°C until used.

RNA extraction and cDNA synthesis

Cultured DRG cells and DRG tissues were homogenized and lysed in 1 ml of TRIzol (Life Technologies) according to the manufacturer’s guidelines. Chloroform (200 μl) was added and the tubes were shaken vigorously for 30 s followed by 2 min of incubation at room temperature. The samples were then centrifuged at 13,000 × g for 20 min at 4°C. The upper phase (450–500 μl) was transferred to a fresh tube. Isopropyl alcohol (500 μl) was added to precipitate the total RNA at room temperature for 10 min followed by centrifuge at 13,000 × g for 20 min at 4°C. The resulting RNA pellets were washed with 75% ethanol. Total RNA was dissolved in diethylpyrocarbonate-treated water. RNA was treated with DNase (2 μg/ml) (Invitrogen) in the presence of RNase out 20 U (Invitrogen) at 37°C for 1 h followed by a 10-min incubation at 70°C, shown to be free of contaminating cellular DNA. cDNA was synthesized using 1 μg of RNA, 5 μl of 10 μM dNTP, 100 ng of random primers (Roche), 200 U of Superscript (Invitrogen) and 20 U of RNase out (Invitrogen). Reactions were performed at 37°C for 90 min, 70°C for 15 min. cDNA was stored at −20°C until used, as previously reported (42).

DNA extraction

The cultured cells were lysed in 200 μl of SDS lysis solution at 50°C for 15 min. Genomic DNA was extracted twice with an equal volume of Tris buffer phenol (pH 8.0) and centrifuged 10 min at 13,000 rpm and once with an equal volume chloroform:isoamyl alcohol 24:1 and centrifuged for 10 min at 13,000 rpm. A one-tenth volume of 3 M sodium acetate (pH 4.8) and 3 vol of 100% ethanol were added, incubated at room temperature for 10 min, and centrifuged 15 min at 13,000 rpm. The pellets were washed with 70% ethanol. Total genomic DNA was dissolved in TE buffer.

Real-time PCR

Genomic DNA and cDNA derived from cultured DRG cultures and DRG tissues were amplified by nested PCR of the viral pol gene as described previously (34). Changes in mRNA levels of specific genes were quantified by real-time PCR using the i-cycler iQ system (Bio-Rad). cDNA prepared from total RNA of cultured cells and DRG tissues was diluted 1/1 with sterile water and 5 μl were used per PCR. The primers used in the real-time PCR were as follows: GAPDH: Forward primer, 5'-AGC TTG CTC CAT GGT GAA-3'; reverse primer, 5'-CGG AGT CAA CGG ATT TGG TG-3'; iNOS: forward primer, 5'-ACT TGG ATC AGA AGT TGG CCC-3'; reverse primer, 5'-CAAG GGT CTG TGA GTC CAT CAC-3' (Tₘ 56°C); STAT-1: forward primer, 5'-CCG GGA AGG GCC CAT CAT AC-3'; reverse primer, 5'-CCA TCT GGC ACA ACA TCT CAA AC-3' (Tₘ 55°C) and TNF-α: forward primer, 5'-CCCC CAG GCC TGC AGA TGG-3'; reverse primer, TGG GGC AGG GGG TTG AGT AGT (Tₘ 55°C). Semi-quantitative analysis was performed by monitoring in real time the increase in fluorescence of SYBR-Green dye. Real-time fluorescence measurements were performed, and a threshold cycle value for each gene of interest was determined, as reported previously (42). All data were normalized to the GAPDH mRNA threshold cycles level and expressed as mRNA relative fold change.

Immunohistochemistry and immunofluorescence

DRG sections on glass slides and 2% PBS-buffered parafomaldehyde-fixed cultured DRG cells plated on chamber slides were incubated with...
PBS containing 50% normal goat serum overnight at 4°C to block nonspecific staining. The sections and slides were exposed either to mouse anti-MAP-2 (clone HM-2, 1/1000 dilution; Sigma-Aldrich), rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba-1) (1/1000 dilution; Wako Chemicals), rabbit anti-glial fibrillary acidic protein (GFAP, 1/1000 dilution; DAKO), mouse anti-CXCR4 (1/100 dilution; R&D Systems), mouse anti-CCR5 (1/50 dilution; BD Pharmingen), mouse anti-iNOS (1/100 dilution; BD Transduction Laboratories), mouse anti-STAT-1 (1/100 dilution; BD Pharmingen), rabbit anti-nitrotyrosine (1/100 dilution; Upstate Biotechnology), mouse anti-CD18 (1/10 dilution; Leukocyte Antigen Laboratory), rabbit anti-activated caspase-3 (1/100 dilution; Trevigen), mouse anti-FIV envelope (1/100) or mouse anti-FIV p24 (1/1000 dilution), obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, overnight at 4°C followed by washing in PBS, then incubating with either HRP-conjugated goat anti-mouse IgG (1/500 dilution), or Cy3 or Alexa 488-conjugated goat anti-rabbit or mouse (1/1000 dilution; Molecular Probes) for 2 h at room temperature in the dark followed by washing in PBS. Subsequently, the chromogenic reaction was conducted on the slides exposed to HRP-conjugated secondary Ab with diaminobenzidine and the reaction was terminated with tap water. The sections and slides were mounted with Gelvatol. The specificity of staining was confirmed by omitting the primary Ab. The sections and slides with immunofluorescent staining were examined on a Zeiss Axioskop 2 Upright Microscope and Spot System (Diagnostic Instruments) provided digital images to quantify neurite length and soma size of neurons using the public domain program Scion Image.

Quantitation of neuronal injury and loss

Neuronal injury was quantified in cultures immunolabeled with MAP-2 Abs at days 2, 4, and 6 postinfection by an examiner unaware of the slide identity. Following completion of the immunolabeling protocol, slides were imaged for subsequent measurements of neuronal soma area, maximal neurite length per neuron, the number of processes per neuron, using a minimum of 25–50 neurons per individual treatment from three separate wells. Using Scion Image Image Analysis software, each parameter was assessed, as previously reported (43). In addition, cell survival was measured in terms of the number of MAP-2-immunopositive neurons per unit area in triplicate. All experiments were repeated at least twice and performed in triplicate.

Western blot analysis

Cultured FIV-infected and mock-infected feline macrophages, extracted in buffer containing 10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl2, and 0.5% Nonidet P-40, was cleared by centrifugation, and protein levels were quantified using a Bradford assay (Bio-Rad). Equal amounts of protein (20 μg/sample), determined by Coomassie blue staining and detection of housekeeping proteins, were separated by SDS-PAGE, transferred to nitrocellulose, and blocked with 5% BSA in TBST (25 mM TBS and 0.1% Tween 20). The primary mAb recognizing p-STAT-1 (Tyr701) (Santa Cruz Biotechnology) was diluted 1/100 in TBST containing 5% BSA, and the primary mAb recognizing STAT-1 (BD Pharmingen) was diluted 1/1000 in TBST containing 5% milk then incubated with membrane, respectively, at room temperature. Following incubation, the membranes were washed and incubated for 1 h at room temperature with HRP-conjugated rabbit anti-goat IgG (Jackson Immunoresearch Laboratories) and HRP-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories) diluted 1/2000 in 5% milk-TBST. Immune-reactive proteins were detected by chemiluminescence (Roche Diagnostics). Stripped, the blot was blocked with 10% milk-TBST 1 h at room temperature, then incubated with HRP-conjugated β-actin (1/2000 dilution; Santa Cruz Biotechnology) 1 h at room temperature. Following the wash with TBST, protein was detected by chemiluminescence (Roche Diagnostics).

Protein carbonyl assay

The level of protein oxidation was determined by an oxidized protein detection kit (Oxyblot; ONCOR) (44). Supernatants collected from FIV-infected or uninfected DRG cultures in presence or absence of AG (0.5 mM) were incubated for 20 min with 12% SDS and 2,4-dinitrophenyhydrazine in 10% trifluoroacetic acid, vortexing every 5 min, and then neutralized with Oxyblot neutralization solution. A total of 250 ng of protein was blotted onto nitrocellulose paper by the slot-blotting technique. Membranes were incubated with blocking buffer for 30 min at room temperature, exposed to rabbit anti-2,4-dinitrophenyhydrazine protein Ab (1/150) for 90 min, followed by anti-rabbit IgG coupled to alkaline phosphatase (1/15,000) for 2 h at room temperature. Following washing and development with SigmaFast chromatogen, blots were analyzed by computer-assisted imaging software, Scion Imaging. Results were expressed as relative fold change over levels in culture medium.

Statistical analysis

Statistical analyses were performed using GraphPad InStat version 3.0 (GraphPad Software). Analysis was performed using the unpaired t test for histopathological changes and real-time RT-PCR analysis. Values of p < 0.05 were considered significant.

Results

FIV infection of ex vivo DRG cultures

As the pathology of HIV-related DSP is characterized by DRG neuronal injury and axonal degeneration (10), we developed an ex vivo system to explore the mechanisms underlying the development of DSP using feline DRGs. The cell types in the DRG cultures were defined by immunolabeling, which revealed that cultures were composed of neurons that were MAP-2 immunopositive (Fig. 1A), GFAP-immunopositive Schwann cells (Fig. 1B), and macrophages that were Iba-1 immunopositive (Fig. 1C). Quantification of cell types present in the cultures revealed mean absolute numbers ±SEM of neurons (141 ± 6.8) and macrophages (1500 ± 63.6) per cm², while Schwann cells were not counted because of their indistinct morphology. Indeed, immunolabeling studies also showed that CCR5 (Fig. 1D) and CXCR4 (Fig. 1E) were detected in DRG cultures and were largely colocalized with Iba-1 immunolabeling (Fig. 1, D and E, insets), although not unexpectedly, some neurons also expressed these chemokine receptors. Infection of the DRG cultures with the neurovirulent FIV
strain, V1-Ch, revealed that infected cultures were FIV p24 immunoreactive (Fig. 2A), which was colocalized with Iba-1 immunoreactivity (Fig. 2B). Conversely, colocalization of FIV p24 expression with GFAP or MAP-2 immunopositive cells was not observed (data not shown). Infected cultures also exhibited FIV envelope immunoreactive cells (Fig. 2C), which resembled macrophages, while mock-infected cultures did not exhibit FIV envelope immunolabeling (Fig. 2D). These latter findings were complemented by PCR analyses of genomic DNA and cDNA derived from control and V1-Ch-infected cultures, which revealed that FIV provirus was detectable at days 2, 4, and 6 postinfection, while viral RNA was evident at days 2 and 4 postinfection only (Fig. 2E). These observations indicated that the present DRG cultures contained all of the cellular components comprising in vivo DRGs and were permissive to FIV infection and replication, albeit limited to macrophages only.

**Neuronal injury in FIV-infected DRG cultures**

Because axonal and neuronal soma injury are key components of DSP, we examined the morphological changes of DRG neurons after FIV (V1-Ch) infection by assessing MAP-2 immunopositive neurons in terms of total neuronal numbers, soma size, and maximal neurite length at days 2, 4, and 6 postinfection. MAP-2 was located in neuronal cell bodies and processes (Fig. 3A), as previously reported (45) but in FIV-infected cultures neuronal process lengths and neuronal perikaryal size were reduced (Fig. 3B). Activated caspase-3 immunoreactivity was evident in some MAP-2-positive neuronal perikarya of FIV-infected cultures (Fig. 3B, inset). Quantitative analyses of neurite length revealed a decline in mean maximal neurite length beginning at day 2 postinfection but continuing until day 6 in the V1-Ch-infected cultures compared with matched mock-infected cultures (Fig. 3C). Similarly, neuronal soma size was also reduced in V1-Ch-infected cultures relative to uninfected cultures at the same time points (Fig. 3D). In fact, there was a progressive atrophy of neuronal perikarya over time postinfection. Likewise, total neuronal perikaryal numbers were significantly reduced in FIV-infected cultures at day 4 (63%) and day 6 (81%), but not at day 2 (97%) postinfection when expressed relative to the time-matched mock-infected cultures (Fig. 3E). Thus, these findings suggested that FIV infection of DRG cultures resulted in neuronal injury, as evidenced by neurite retraction, neuronal cell body atrophy and fewer surviving neurons, some of which exhibited evidence of apoptosis.

**FIV induces STAT-1 and iNOS in DRG macrophages**

Previous studies have shown that the HIV mediates the induction of STAT-1 expression in macrophages (46, 47), which was recapitulated in further studies by showing that STAT-1 expression was up-regulated in a HIV or a FIV envelope sequence-dependent manner (34, 35). The FIV strain, V1-Ch, induced STAT-1 most significantly.
efficiently in these latter studies and thus, we focused on this virus because it was also most neurovirulent in the present ex vivo assays. To determine whether these STAT-1-associated findings also applied to the peripheral nervous system following lentivirus infection, we examined in vivo changes in DRGs from FIV-infected and control animals from previously reported studies (19). Importantly, DRGs from FIV-infected animals exhibited both FIV provirus and viral RNA (Fig. 4A), similar to our earlier findings in nerves from FIV-infected animals (19). This finding was complemented by immunofluorescent studies, which showed that FIV envelope immunoreactivity was detectable in both nucleus and cytoplasm of macrophages within DRGs (inset represents merged STAT1 and Iba-1 images). F. Minimal STAT-1 immunoreactivity was present in DRGs from mock-infected cats. G, iNOS mRNA levels (mean ± SEM) and protein levels (H) were higher in DRGs from FIV-infected cats (n = 4) compared with mock-infected cats (n = 4) (I). iNOS immunoreactivity was detectable in Iba-1 immunopositive macrophages (H; inset shows colocalization) (original magnification: B, C, E, F, H, and I, ×200; inset, ×400; n = 4 for each group; *, p < 0.05).

FIGURE 5. Expression of STAT-1, iNOS, TNF-α in FIV (V1-Ch)-infected cultures. mRNA levels (mean ± SEM) for (A) STAT-1, (B) iNOS and (C) TNF-α were measured in FIV-infected cultures, relative to mock-infected cultures. Western blotting confirmed higher total STAT-1 and p-STAT-1 protein levels in FIV-infected macrophages compared with mock-infected macrophages at days 1 and 2 postinfection (D) (*, p < 0.05).
controls (Fig. 4D) and subsequent studies showed that STAT-1 immunoreactivity was detected in the nucleus and cytoplasm of Iba-1 immunopositive macrophages within the DRG (Fig. 4E; inset shows colocalization) while there was minimal detection in DRGs from mock-infected animals (Fig. 4F). Because STAT-1 is known to transactivate the iNOS gene through its IFN-γ-activated site (48), we also examined the expression of iNOS in DRGs from control and FIV-infected animals. These investigations revealed that iNOS mRNA levels were increased in DRGs from FIV-infected animals compared with uninfected animals (Fig. 4G). iNOS immunoreactivity was detectable in Iba-1 immunopositive macrophages (Fig. 4H; inset shows colocalization) and in some Schwann cells from FIV-infected animals, while there was minimal expression in DRGs from uninfected animals (Fig. 4I). Thus, these studies reflected findings in the CNS following lentivirus infection in terms of induction of STAT-1 and iNOS in monocyte/macrophages (34, 49).

These latter studies were extended to the ex vivo DRG model, which revealed that STAT-1 mRNA levels were induced at day 2 postinfection in V1-Ch infected DRG cultures relative to mock-infected cultures (Fig. 5A) but not at days 4 and 6 postinfection. Similarly, iNOS mRNA was induced at days 2 and 4 postinfection in FIV-infected DRG cultures compared with uninfected cultures (Fig. 5B). In keeping with earlier in vivo studies from our laboratory (19), TNF-α expression was increased at days 2, 4, and 6 postinfection in FIV-infected ex vivo DRG cultures compared with mock-infected cultures (Fig. 5C). Because our in vivo DRGs showed STAT-1 expression in macrophages, we examined STAT-1α protein levels in FIV-infected macrophages, which showed both increased STAT-1 and phosphorylated STAT-1 at days 1 and 2 after FIV infection, compared with mock-infected macrophages (Fig. 5D). However, comparisons of proteinase-activated receptor-2 expression in DRG cultures did not differ between infected and uninfected cultures at any time points (data not shown). Hence, these findings suggested that FIV infection results in selective up-regulation of STAT-1 and iNOS, largely in cells of monocyte/macrophage lineage following FIV infection of DRG cultures.

AG prevents redox reactant production and neuronal injury

Previous reports have suggested that up-regulation of iNOS is associated with increased protein nitrosylation and free radical production predominantly through the production of NO and its redox reactants (29, 50, 51). To explore this possibility in the current model, we examined the expression of 3-nitrotyrosine (3NT). 3NT immunoreactivity was increased in FIV-infected cultures (Fig. 6A), largely detected on macrophages (Fig. 6A, inset), compared with uninfected cultures (Fig. 6B). To determine whether this finding was related to iNOS induction, DRG cultures were treated with the iNOS inhibitor, AG, which showed that in AG-treated cultures there was minimal detection of 3NT in both FIV-infected (Fig. 6C) and uninfected cultures (Fig. 6D). Quantitation of 3NT immunopositive cells revealed similar findings of markedly increased 3NT levels in the FIV-infected cultures compared with controls (Fig. 6E) and in contrast, 3NT levels were significantly reduced in the AG-treated FIV-infected cultures (Fig. 6E). These findings were complemented by accompanying studies, which showed that protein carbonyl levels, an index of protein oxidation, in culture supernatants were increased in FIV-infected cultures relative to mock-infected cultures (Fig. 6F), while AG reduced protein carbonyl abundance, indicating that AG inhibited the production of 3NT and the oxidation of cellular protein components.

As neuronal injury is a key feature of lentivirus infection of DRG, we also examined the effects of AG and a specific iNOS inhibitor, l-NIL on neuronal injury, which revealed that both compounds protected FIV-infected DRG cultures in terms of neurite...
Finally, overall neuronal survival was also markedly impaired in FIV-infected cultures, relative to FIV-infected untreated cultures (Fig. 7B). These findings underscore the importance of iNOS induction with the production NO, resulting in neuronal injury within the DRG.

**Discussion**

This report represents the first mechanistic investigation of DSP pathogenesis using an infectious virus model, which recapitulates HIV-related DSP. Our findings point to a very specific disease process that has not been described previously in the peripheral nervous system to date. The shared features of the ex vivo model of DRG injury caused by infection of a neurovirulent molecular clone of FIV (V1-Ch) and in animals infected by the same virus serve to emphasize the value of the model, while providing new insights in DSP pathogenesis at the same time. Although the DRG cultures consisted of Schwann cells, macrophages, and neurons that were vulnerable to cytotoxic factors, only the macrophages were productively infected by FIV with resulting neuronal injury manifested as neurite retraction, reduced soma size, and neuronal depletion. Accompanying FIV infection of the cultures was evidence of specific host immune responses including activation of STAT-1, iNOS, and TNF-α, which were also apparent in DRGs from FIV-infected animals. Inhibiting iNOS activity with AG or l-NIL protected neurons from FIV’s pathogenic effects, further underscoring the importance of NO in the pathogenesis of neuroinflammation in the peripheral nervous system.

Neuronal injury and axonal dying back are signature features of HIV-related DSP, likely contributing to the clinical manifestations of DSP. Damage and loss of DRG neurons were also central features of the present studies with evidence of apoptosis. Neuronal loss may have resulted from neurotoxins released from activated macrophages or the loss of trophic support by damaged Schwann cells. Indeed, Schwann cells may also be targets for cytotoxins released by FIV-infected or activated macrophages, consistent with reports of HIV infection of the CNS in which astrocyte apoptosis is correlated with the development of HIV-associated dementia and neuronal loss (52). However, Schwann cells did not exhibit productive FIV infection, similar to neurons, which are also not infected by FIV (53). It is also plausible that sensory perikaryal dysfunction in FIV-infected animals results in failure to elaborate and export structural proteins in nerve including neurofilament and tubulin that are required to maintain axon caliber and integrity, manifesting as neurite retraction in the present studies.

The underlying pathogenesis for HIV-1 DSP is undefined but several potential contributing factors have been identified in the past including chronic inflammation involving HIV-activated and infected macrophages within peripheral nerves, together with advanced systemic immunosuppression. The DRG neuron may be especially vulnerable to injury during HIV-1 infection because of the relatively permeable blood-nerve barrier within the DRG, compared with other regions of the peripheral nerve. Macrophage activation and enhanced TNF-α expression was evident in our previous studies of peripheral nerves from FIV-infected animals (19). Similarly, we observed increases in expression of the proinflammatory cytokine TNF-α in ex vivo FIV-infected DRG cultures. Accompanying this finding was the increase in STAT-1 and iNOS expression in both in vivo and ex vivo FIV-infected DRGs. Of interest, recent studies suggest that HIV infection and HIV gp120 treatment of thymus cultures also activate STAT-1 production, which are analogous to earlier studies from our group (34) and others (35, 54), showing that both the FIV and HIV envelopes drive STAT-1 expression. These studies emphasize the importance the lentivirus envelope as a critical pathogenic determinant. However, it is likely that other host genes are overexpressed in DRGs of FIV-infected animals including proinflammatory cytokines and chemokines, which modulate the expression of neuropathogenic effectors such as NO with ensuing neuronal damage.

iNOS expression has been reported to be increased in several inflammatory disorders of the peripheral nervous system in both the DRG and nerve with conflicting effects including neuroprotective and neuropathogenic properties (36, 55, 56). Because macrophages express CCR5, one of the coreceptors for FIV, often together with STAT-1 (40), and the natural ligand for CCR5, RANTES, induces the phosphorylation of STAT-1 (57), it was not surprising to see up-regulation of iNOS in the present studies. In fact, iNOS expression is regulated by STAT-1 through the IFN-γ-activated site domain located in the iNOS promoter (58). iNOS expression has been extensively described in rodent monocytoid cells but less well in other species although several studies have suggested that iNOS is up-regulated in human macrophages infected by HIV (59, 60). Moreover, inhibiting iNOS with AG exerted a neuroprotective effect in DRG cultures infected by FIV. This latter observation is similar to studies of CNS neuronal injury in which neurons are protected in select diseases by AG (61) while...
in the peripheral nervous system, the neuroprotective properties of AG are more likely to be due its effects on blood flow because of its nonspecific effects on different NOS isoforms (33). Indeed, our findings with AG as reinforced by the similar neuroprotective effects mediated by the iNOS-specific inhibitor, l-NIL. Neuroprotection mediated by inhibition of iNOS, likely reflects diminished ONOO− synthesis or peroxynitrite-induced nitrosylation of Src homology moieties, as previously reported (62).

FIV is one of several models of HIV-1 infection (53). The principal asset of this model is the use of a small animal which displays progressive immunosuppression together with neurodegenerative disease including encephalopathy, polymyositis, and distal sensory polyneuropathy, recapitulating primary neurodegenerative complications of HIV-1 infection (63, 64). The FIV envelope engages both CXCR4 and CCR5 for infection, depending on the viral strain, similar to HIV-1 but FIV does not use CD4 as its primary receptor. In fact, there is evidence that HIV-1 infection also occurs independent of CD4 in vivo and in vitro, as reported for other principal HIV animal models including SIV (53). The primary receptor for FIV is uncertain although CD134 has recently been postulated to be a candidate molecule (65); however, immunolabeling in the present studies did not identify CD134 on DRG macrophages, Schwann cells or neurons (data not shown). Nonetheless, the current studies enlarge FIV’s use as an HIV model by presenting an ex vivo approach consisting of the cellular components within DRGs including monocyteid cells that are permissive to infection by FIV. Importantly, macrophage tropism is a cardinal feature of all lentivirus infections (15, 66, 67).

The infectious recombinant FIV clone, V1-Ch, used in the present studies was found to be highly neurovirulent in earlier studies (19, 34). Neurovirulence is likely dependent on both input titer (68) together with the intrinsic virulence encoded by the FIV envelope (18) and the resulting systemic immunosuppression. To date, little is known about the biological features including cell tropism, viral quantity (load) and coreceptor dependence of peripheral nerve-derived virus in HIV-1 infection. Very recently, we have shown that peripheral nerve-derived HIV-1 is largely CCR5-dependent and macrophage tropic although viral burden in human peripheral nerve with DSP was low (69). Presumably, both HIV-1 and FIV engage their cognate receptors on macrophages for infection of DRGs and peripheral nerves leading to infection and macrophage activation. However, given the prominence of viral burden in human immunodeficiency virus-infected patients: incidence and relationship to other nervous system dysfunction. Arch. Neurol. 48: 1273–1274.


