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Atrophy and Death of Nonpeptidergic and Peptidergic Nociceptive Neurons in SIV Infection

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HIV-associated sensory neuropathy is a common neurologic comorbidity of HIV infection and prevails in the post-antiretroviral therapy (ART) era. HIV infection drives pathologic changes in the dorsal root ganglia (DRG) through inflammation, altered metabolism, and neuronal dysfunction. Herein, we characterized specific neuronal populations in an SIV-infected macaque model with or without ART. DRG neuronal populations were identified by neurofilament H-chain 200, I-B₄ isolectin (IB₄), or tropomyosin receptor kinase A expression and assessed for cell body diameter, population size, apoptotic markers, and regeneration signaling. IB₄⁺ and tropomyosin receptor kinase A-positive neurons showed a reduced cell body size (atrophy) and decreased population size (cell death) in the DRG of SIV-infected animals compared with uninfected animals. IB₄⁺ nonpeptidergic neurons were less affected in the presence of ART. DRG neurons showed accumulation of cleaved caspase 3 (apoptosis) and nuclear-localized activating transcription factor 3 (regeneration) in SIV infection, which was significantly lower in uninfected animals and SIV-infected animals receiving ART. Nonpeptidergic neurons predominantly colocalized with cleaved caspase 3 staining. Nonpeptidergic and peptidergic neurons colocalized with nuclear-accumulated activating transcription factor 3, showing active regeneration in sensory neurons. These data suggest that nonpeptidergic and peptidergic neurons are susceptible to pathologic changes from SIV infection, and intervention with ART did not fully ameliorate damage to the DRG, specifically to peptidergic neurons. (*Am J Pathol* 2020, 190: 1530–1544; <https://doi.org/10.1016/j.ajpath.2020.03.004>)

With the introduction of combined antiretroviral therapy (ART), HIV infection has transitioned to a chronic disease, shifting focus to HIV-associated comorbidities, persistent inflammation, and early-onset age-related illnesses. HIV-associated sensory neuropathy remains one of the most common neurologic complications of HIV infection, significantly contributing to HIV morbidity with little reduction in prevalence from ART.^{1–4} The most common form of HIV-associated sensory neuropathy, HIV-associated distal sensory polyneuropathy (HIV-DSP), develops from damage to peripheral sensory neurons of the dorsal root ganglia (DRG) and the dying back of the primary afferent axon terminals.^{5–7} HIV-DSP manifests with pain, numbness, tingling sensations, and temperature hypersensitivity in the extremities as a result of abnormal peripheral neuron function; however, the pathophysiology

of neuronal damage and dysfunction is multifaceted and not fully understood.

The DRG are located adjacent to the spinal column and consist of a heterogeneous population of somatosensory neurons responsible for conveying peripheral sensations to the central nervous system through innervating the dorsal horn of the spinal cord. Of particular interest in HIV-DSP,

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C-fiber nociceptive neurons make up most of the somatosensory neurons in the DRG and are responsible for conveying pain, temperature, itch, and touch.^{8–10} Nociceptive neurons have been classified as non-peptidergic, identified as I-B₄ isolectin (IB4) binding neurons, and peptidergic neurons, expressing tropomyosin receptor kinase A (TrkA) and neuropeptides, such as calcitonin gene-related peptide.^{10–13} Nociceptive afferents in cutaneous tissue are mostly unmyelinated and branch into the epidermal area as free-end terminals.^{14–17} Innervating free axon terminals are particularly susceptible to changes in inflammatory status, as they can directly interact with macrophages, neutrophils, mast cells, Merkel cells, and blood vessels in the skin. In the presence of pathologic changes and disease, nerve fibers die back from the epidermal layer as a result of damage to terminals, axons, and cell bodies.

DRG neurons are long-lived neurons with high axonal regenerative capacity and resilience to environmental changes; however, death of DRG neurons is permanent. In the presence of HIV infection, neurons have diminished regenerative capacity, impaired pain transmission, and abnormal metabolic function, all contributing to loss of axon terminals innervating the periphery.^{18–21} Damage to peripheral neurons is accompanied with significant transcriptional changes in survival genes and regeneration-associated genes.^{22,23} An immediate-early gene in the regenerative program is activating transcription factor 3 (ATF3). The increased expression and nuclear accumulation of ATF3 is integral to the initiation and maintenance of the regenerative state of peripheral neurons.^{24–27} ATF3 was found to be a critical mediator of regeneration-associated gene expression in sensory neurons and promoted significant neurite regrowth.^{28,29} Priming neurons for regeneration is dependent on transcriptional changes from multiple, converging pathways and vital for sensory neuron survival in challenge.

The extent of damage to sensory neurons remains unclear in the context of HIV infection. Previous studies in animal models have detailed the presence of viral proteins in the DRG, satellite cell activation, increased infiltration of mononuclear cells into the DRG, and increased production of inflammatory mediators in the DRG, detailing a prominent role of inflammation in development of pathology.^{18,21,30–32} However, specific neuronal population changes and damage responses have yet to be investigated. The SIV-infected rhesus macaque model was used to characterize alterations in different sensory neuron populations, the induction of regeneration transcription factors, and activation of apoptosis in the DRG, to infer neuronal contributions to and exacerbation of the clinical manifestations of HIV-DSP. A significant, class-specific atrophy and loss of nociceptive sensory neurons was observed with SIV infection. Both proregenerative, stress-induced signaling (ATF3) and apoptotic signaling (cleaved caspase 3) was observed in DRG neurons during SIV infection. Herein, we show dramatic changes in DRG neurons that suggest, in

addition to inflammatory and viral alterations in the DRG, the presence of SIV infection promotes a severe pathologic environment for nociceptive sensory neurons.

Materials and Methods

Animals, Viral Infection, CD8 T-Cell Depletion, and ART Regimen

Indian, male rhesus macaques (*Macaca mulatta*) were used in this study as a model of HIV infection (Table 1).^{33–36} Primates are recognized as an acutely scarce resource and as such are specifically exempted from the requirement of sex balancing; herein, we conducted the study in an all-male cohort (NIH: NOT-OD-15-102). Animals were housed at either the Tulane National Primate Research Center (Covington, LA) or the New England Regional Primate Center. Animals A01 to A08 served as uninfected controls for the study (referred to as SIV[−]; *n* = 9). Two SIV[−] animals were CD8 depleted (A01 and A02) and six were not CD8 depleted (A03 to A08). Twenty animals were inoculated intravenously (i.v.) with SIVmac251 viral swarm (5 ng p27; Tulane National Primate Research Center's Viral Core) and subsequently CD8 depleted through administration of 10 mg/kg of anti-CD8 antibody subcutaneously at 6 days postinfection (dpi) and 5 mg/kg of antibody intravenously at 8 and 12 dpi (Nonhuman Primate Reagent Resource), as previously described.^{21,30,37} Animals A09 to A11 were time sacrificed at 21 dpi on establishment of viral infection (referred to as SIV/DPI21; *n* = 3). Animals A12 to A22 did not receive a treatment regimen and progressed to terminal SIV and simian AIDS (referred to as SIV/AIDS; *n* = 11). The development of simian AIDS was determined postmortem, as previously described.^{21,30,37} Animals A23 to A28 received a clinically relevant ART regimen of raltegravir (22 mg/kg orally twice daily; Merck, Kenilworth, NJ), tenofovir (30 mg/kg subcutaneously once daily; Gilead, Foster City, CA), and emtricitabine (10 mg/kg subcutaneously once daily; Gilead) at 21 dpi until the timed sacrifice at 118 to 120 dpi (referred to as SIV + ART; *n* = 6).^{38,39} A representative experimental design details SIV inoculation, CD8 depletion, and ART administration for all animals (Supplemental Figure S1). All animals were anesthetized with ketamine-HCL and euthanized by i.v. pentobarbital overdose. These animals were sacrificed according to humane end points consistent with the recommendations of the American Veterinary Medical Association *Guidelines for the Euthanasia of Animals*, as previously described.^{21,37,40} Animals were necropsied immediately following death, and representative sections of all major organs were collected for histologic analysis. All animals used in this study were handled in strict accordance with American Association for Accreditation of Laboratory Animal Care with the approval of the Institutional Animal Care and Use Committee of Harvard University and the Institutional Animal Care and Use Committee of Tulane University.

Table 1 Viral, Pathologic, and Zoologic Information of Animals Used in the Study

Group I.D.	Animal I.D.	Age, years	Primate center	Survival, days	Plasma VL, log ₁₀ copies/mL*	DRG pathology (score [†])
Uninfected (SIV–)	A01	6.0	NERPC	N/A	N/A	Normal (0)
	A02	6.1	NERPC	N/A	N/A	Normal (0)
	A03	10.8	TNPRC	N/A	N/A	N/A
	A04	9.6	TNPRC	N/A	N/A	N/A
	A05	6.7	TNPRC	N/A	N/A	N/A
	A06	7.9	TNPRC	N/A	N/A	N/A
	A07	7.7	TNPRC	N/A	N/A	N/A
	A08	3.3	TNPRC	N/A	N/A	N/A
SIV infected and CD8 depleted (SIV/DPI21)	A09	9.1	TNPRC	21	6.87	Moderate (2)
	A10	5.2	TNPRC	21	7.15	Moderate (2)
	A11	5.3	TNPRC	21	6.84	Mild (1)
SIV infected and CD8 depleted (SIV/AIDS)	A12	10.4	TNPRC	89	7.71	Moderate-severe (2.5)
	A13	7.3	TNPRC	55	7.83	Mild-moderate (1.5)
	A14	10.8	TNPRC	174	7.28	Severe (3)
	A15	11.5	TNPRC	146	7.67	Severe (3)
	A16	3.8	NERPC	77	7.23	Moderate-severe (2.5)
	A17	5.8	NERPC	77	8.54	Moderate (2)
	A18	4.3	NERPC	168	5.88	Mild (1)
	A19	5.4	NERPC	97	7.79	Mild (1)
	A20	6.3	TNPRC	84	5.41	Severe (3)
	A21	4.5	TNPRC	96	7.15	Mild (1)
	A22	7.3	TNPRC	106	7.69	Moderate (2)
	A23	6.1	TNPRC	118	2.66	Mild (1)
	A24	6.7	TNPRC	118	2.66	Mild (1)
	A25	6.4	TNPRC	119	1.60	Mild (1)
	A26	10.3	TNPRC	119	3.62	Mild (1)
SIV infected and ART treated (SIV + ART)	A27	6.2	TNPRC	120	2.34	Mild (1)
	A28	10.4	TNPRC	120	2.87	Mild (1)

All animals in the study were male, Indian rhesus macaques.
*Plasma VL was measured at necropsy as terminal viral load.
[†]General scoring includes lumbar and sacral DRG for pathology score. Pathologic grading and criteria based on evaluation from a certified veterinary pathologist (refer to *Materials and Methods*).
DRG, dorsal root ganglia; I.D., identifier; N/A, not applicable; NERPC, New England Regional Primate Center; Plasma VL, plasma viral load, at necropsy; TNPRC, Tulane National Primate Research Center.

Pathologic Assessment

Tissues were evaluated for pathologic changes in the DRG through hematoxylin and eosin staining. Sections were evaluated blindly by a board-certified veterinary anatomic pathologist (A.D.M.) and scored on the basis of the presence and severity of infiltrating mononuclear cells, neuronophagia, and Nageotte nodules, as previously described.^{21,30,31,33} Overall pathology was scored on a previously validated scale of 1 to 3 at increments of 0.5 via the following criteria: i) mild, scattered infiltrating mononuclear cells with rare evidence of neuronophagia and/or neuronal loss; ii) moderate, increased numbers of infiltrating mononuclear cells with occasional neuronophagia and/or neuronal loss; and iii) severe, abundant infiltrating mononuclear cells and frequent neuronophagia and neuronal loss were all present.^{21,30,31,33}

Immunohistochemistry

Tissues were prepared and divided into sections, as previously reported.^{21,30,31,33} Sections were boiled in antigen unmasking buffers for epitope retrieval (Vector, Burlingame, CA; Dako, Carpinteria, CA; and Perkin Elmer, Waltham, MA) and blocked for appropriate endogenous proteins (horseradish peroxidase, alkaline phosphatase, avidin, and biotin). Tissues were incubated in primary antibodies or biotinylated lectins for 1 hour at room temperature (Table 2). Primary antibodies or lectins were detected through horseradish peroxidase–conjugated secondary antibodies (Dako) or avidin-biotin complex (Vector). Target proteins were visualized through the 3,3′-diaminobenzidine chromogen system (Dako), and sections were counterstained with hematoxylin (Sigma, St. Louis, MO). Tissue sections were imaged using the Keyence BZ-X700 microscope in bright

Table 2 Antibodies Used in Histologic Analysis

Target	Antibody isotype (clone)	Company
CD68	Mouse (KP1)	Dako (M0814)
CD163	Mouse (EDHu-1)	Bio-Rad, Hercules, CA (MCA1853)
Mac387	Mouse (Mac 387)	Dako (M0747)
BrdU	Mouse (Bu20a)	Dako (M0744)
CD3	Rabbit	Dako (A0452)
NF200	Mouse (N52)	Millipore, Burlington, MA (MAB5266)
TrkA*	Rabbit	Abcam, Cambridge, UK (ab76291)
ATF3	Rabbit	Sigma (HPA001562)
Cleaved caspase 3	Rabbit	Cell Signaling, Danvers, MA (9661)
Isolectin B4	Lectin†	Vector (B-1205)

*TrkA required High pH Target Retrieval (Dako) for antigen unmasking.

†Not an antibody, Biotinylated Griffonia Simplicifolia Lectin I (GSL I) IB4. ATF3, activating transcription factor 3; BrdU, bromodeoxyuridine.

field for 3,3'-diaminobenzidine chromogenic development (Keyence, Osaka, Japan). Histologic stains were quantified using the Keyence BZX Advanced Analysis Software. Hematoxylin was used to determine total number of neurons in individual images, and identification of positive neurons was achieved by threshold of staining intensity through BZX Hybrid Cell Count (Keyence). Quantifications were reported in percentages of total number of neurons (rather than cells/mm²) to account for differences in number of neurons in each section of lumbar DRG. For each animal, at least eight representative images were quantified for percentage positive cells and averaged to give a representative mean.

RNAscope *in Situ* Hybridization

Caspase 3 RNA in DRG neurons was visualized using RNA-scope,³⁷ according to specifications of the manufacturer (ACDBio, Newark, CA; reference number 43656). Slides were deparaffinized through xylene, washed in 100% ethanol, and air dried. Sections were treated with heat-induced target retrieval (92°C to 100°C) and incubated with protease (40°C). The probe was hybridized in a humidity chamber at 40°C for 2 hours. Caspase 3 RNA was detected by amplification and chromogenic development using the Alkaline Phosphatase—Red Chromogen kit (ACDBio). Sections were counterstained with hematoxylin, dried at 60°C, and mounted. Slides were imaged for quantification of RNA signal using Texas-Red filter fluorescence with a Keyence BZ-X700 Microscope. Threshold level of detection for RNAscope was determined through Dap8 negative control probes (bacterial gene) and PPIB positive control probe (low-expression constitutive gene).

Immunofluorescence

Detection of multiple target proteins was done through immunofluorescence using Opal dyes, according to

manufacturer's specifications (Perkin Elmer). Paraffin sections were deparaffinized and rehydrated through graded ethanols. Sections were boiled in AR6 (neutral pH) or AR9 (high pH) retrieval buffer (Perkin Elmer), then blocked and incubated with primary antibody at room temperature for 1 hour (Table 2). Primary antibodies or lectins were detected by horseradish peroxidase—conjugated dual mouse-rabbit secondary antibody or avidin-biotin complex, respectively. Antibody complexes were visualized with Opal dyes for 520-, 570-, or 690-nm wavelength. Slides were then heat treated with AR6 or AR9 retrieval buffer to reduce background, strip sections of primary and secondary antibody, and allow restaining for other target proteins. Following detection of all targets, slides were counterstained with spectral DAPI (Perkin Elmer) and mounted with Prolong Gold antifade mounting media (Vector). Slides were imaged using filter cubed immunofluorescence with the BZ-X700 microscope (Keyence). Positive staining was determined by setting a threshold of detection compared with negative controls using Keyence Hybrid Cell Count. The total count of a neuronal population and overlap were determined using Keyence BZX Advanced Analysis Software. The number of double-positive neurons and total number of the neuronal population of interest were counted and used to determine the percentage double-positive neurons. For a magnification of $\times 20$ (ATF3), at least eight representative images were quantified for percentage positive cells and averaged to give a representative median. For a magnification of $\times 60$ (cleaved caspase 3), at least 20 representative images were quantified for percentage positive cells and averaged to give a representative median.

Neuronal Diameter Measurements

Tissue sections were stained for neurofilament H-chain 200 (NF200), IB4, and TrkA, imaged at $\times 400$ magnification, and stitched to form a representative image of the areas containing all neuronal bodies of the DRG section. Neuronal diameter was blindly assessed to measure all positive neuronal bodies that contained the neuronal nucleus (J.A.R.), and similarly to immunohistochemistry, the neuronal stain threshold was determined by the Keyence BZX Hybrid Cell Count. The diameter was measured from the longest point-to-point distance of the neuronal body using the Keyence BZX Advanced Analysis Software Measurement Module. The average neuronal diameter was determined for each neuronal population as a composite average of all neurons in the section of lumbar DRG. Neuronal diameters were segregated into 5- μ m bins, and the frequency of diameters was determined within each 5- μ m increment.

Enzyme-Linked Immunosorbent Assay

EDTA plasma was used to determine concentrations of inflammatory biomarkers over the course of SIV infection and treatment. Plasma soluble CD163 (IQ Products, Groningen,

the Netherlands), soluble CD14 (R&D Systems, Minneapolis, MN), chemokine (C-C motif) ligand (CCL) 2 (R&D Systems), and CCL5 (R&D Systems) were measured by enzyme-linked immunosorbent assay, according to manufacturer's instructions. Measurements were run in duplicate with appropriate positive controls and accepted with a percentage CV of $\leq 25\%$; samples were repeated if the cutoff was exceeded.

Skin Punch Biopsy and IENFD

Skin punches (3 mm thick) were taken serially near the sural innervation site just distal to the lateral malleolus. Biopsy specimens were taken for each animal at pre-infection and at various time points after infection to necropsy. Biopsy specimens were processed, as previously described, and evaluated for the degree of innervation.^{18,20,21,30,41} The quantitation delivers a measure of nerve fiber length/volume epidermis [intraepidermal nerve fiber density (IENFD)].

BrdU Administration

Bromodeoxyuridine (BrdU) was prepared as previously reported.^{21,37} BrdU was administered by slow bolus i.v. injection at a dose of 60 mg BrdU/kg body weight. BrdU was administered at several time points over the course of infections and 24 hours before necropsy in terminal SIV/AIDS and SIV + ART animals (A12 to A28).

Flow Cytometry

Flow cytometric analyses were performed with 100- μ L aliquots of EDTA-coagulated whole blood, as previously reported.^{21,42,43} Erythrocytes were lysed using ImmunoPrep Reagent System (Beckman Coulter, Brea, CA), washed twice with phosphate-buffered saline containing 2% fetal bovine serum, and then incubated for 15 minutes at room temperature with fluorochrome-conjugated surface antibodies. Samples were acquired on a BD FACS Aria (BD Biosciences, San Jose, CA) and analyzed with Tree Star Flow Jo version 9.6 (BD Biosciences).

Viral Load Measurement

Plasma SIV-RNA was quantified using real-time PCR for all animals used in this study, as previously described.⁴⁴ The threshold sensitivity was 15 copy Eq/mL, with an average interassay %CV of $<25\%$.

Statistical Analysis

All statistics and graphical representations were done using GraphPad Prism version 8.0 (GraphPad, San Diego, CA). Because of small numbers of animals in groups, all comparisons did not assume a gaussian distribution, and a statistical probability value of $P < 0.05$ was set for the

significance level. All numerical values reported in tables were represented by the median and the interquartile range. All correlations were Spearman correlations and reported with the corresponding r value, if significant. Bar graphs are representative of the median value with the interquartile range. A U -test was used in comparisons of two, nonpaired groups. A Kruskal-Wallis test (nonparametric analysis of variance; referred to as analysis of variance in *Results* and figures) was used to compare three or more, nonpaired groups and, if significant, was followed by a post-hoc Dunn multiple comparison test. Diameter distributions were compared by two-way analysis of variance and, if the interaction was significant, multiple comparisons were tested with a Bonferroni post-test (adjusted for multiple comparisons).

Results

Animals Used in the Study

All animals in the study were male, Indian rhesus macaques (*Macaca mulatta*) (Table 1). Several animals served as uninfected controls in the study (SIV; A01 to A08; $n = 9$). SIV-infected animals sacrificed at 21 days postinfection (SIV/DPI21; A09 to A11; $n = 3$) served as an early time point of established viremia and for comparison for pre-ART initiation. SIV-infected animals that progressed to simian AIDS (SIV/AIDS; A12 to A22; $n = 11$) provided insight into changes in sensory neurons at a terminal stage of SIV/AIDS. SIV-infected animals that received ART, initiated at 21 days postinfection following the establishment of viremia, were treated with a clinically relevant ART regimen until 118 to 120 days postinfection (SIV + ART; A23 to A28; $n = 6$). Plasma viral load of SIV + ART animals was successfully decreased several logs by necropsy; however, more important, the viral load was not fully suppressed to undetectable levels and had a median log₁₀ copies/mL of plasma of 2.66 [interquartile range (IQR) = 0.90] (Table 3 and Supplemental Figure S2). Animals were sacrificed and necropsied for comparison of changes in the DRG as a result of SIV infection.

ART Improves Myeloid Inflammation, DRG Pathology, and DRG Leukocyte Infiltration from SIV Infection

SIV infection generates a severe inflammatory state in the absence of ART. With successful viral load reduction by ART, the necropsy plasma concentrations of sCD14, CCL2, and CCL5 were significantly lower in SIV + ART animals compared with SIV/AIDS animals (plasma markers; $P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively) (Table 3). Plasma sCD163 trended toward a lower plasma concentration in SIV + ART animals compared with SIV/AIDS animals (plasma markers; $P = 0.06$) (Table 3). The percentage monocytes trended to a lower percentage of peripheral blood mononuclear cells in SIV + ART animals compared

Table 3 Comparison of Plasma Immune Markers, IENFD, and LDRG Immune Cells of SIV/AIDS and SIV + ART Cohort

Variable	SIV/AIDS		SIV + ART	
	<i>n</i>	Median (IQR)	<i>n</i>	Median (IQR)
Plasma markers				
Necropsy viral load, log ₁₀ copies/mL	11	7.67 (0.64)	6	2.66 (0.90)***
Soluble CD14, ng/mL	11	1358.2 (253.8)	6	707.2 (216.2)*
Soluble CD163, ng/mL	11	412.4 (230.8)	6	268.2 (103.4)
CCL2, pg/mL	11	719.3 (912.5)	6	118.6 (125.7)**
CCL5, pg/mL	11	5345.0 (36,675.0)	6	2215.2 (2085.4)*
Peripheral blood monocytes and expression [†]				
Monocytes, %	8	5.3 (6.9)	6	4.0 (2.6)
CD14 ⁺ CD16 ⁺ monocytes, %	8	22.3 (19.4)	6	6.6 (5.9)**
CD14 ⁺ CD16 ⁺ monocytes, %	8	9.3 (10.4)	6	4.5 (3.9)*
CD14 ⁺ CD16 ⁺ monocytes, %	8	63.7 (28.2)	6	88.8 (9.7)**
CD163 expression on CD14 ⁺ CD16 ⁺ monocytes, MFI	8	22,543.0 (18,582.8)	6	4751.0 (3659.0)**
IENFD measurements				
Change from pre-infection to necropsy, %	11	-43.3 (37.0)	6	26.1 (125.1)*
Lumbar DRG immune cell counts, cells/mm ²				
CD68 ⁺ macrophages	11	1318.0 (637.7)	6	1395.9 (427.5)
CD163 ⁺ macrophages	11	1306.0 (831.4)	6	659.9 (395.0)*
Mac387 ⁺ monocytes	11	156.7 (93.0)	6	26.3 (40.4)***
BrdU ⁺ monocytes [†]	8 [†]	96.2 (72.8)	6	33.5 (23.5)**
CD3 ⁺ T cells	11	152.2 (418.4)	6	200.1 (89.8)

P* < 0.05, *P* < 0.01, and ****P* < 0.001 for SIV/AIDS versus SIV + ART (nonparametric *U*-test).

[†]No flow cytometry or BrdU data available for animals A20 to A22.

BrdU, bromodeoxyuridine; CCL, chemokine (C-C motif) ligand; DRG, dorsal root ganglia; IENFD, intraepidermal nerve fiber density; IQR, interquartile range; LDRG, lumbar DRG; MFI, mean fluorescent intensity.

with SIV/AIDS animals (peripheral blood monocytes and expression; *P* = 0.08) (Table 3).

The percentage intermediate CD14⁺CD16⁺ monocytes, nonclassic CD14⁺CD16⁺ monocytes, and classic CD14⁺CD16⁺ monocytes significantly differed in SIV-infected animals compared with SIV + ART animals (peripheral blood monocytes and expression; *P* < 0.01, *P* < 0.05, and *P* < 0.01, respectively) (Table 3). Intermediate and non-classic monocytes were significantly lower in SIV + ART animals compared with SIV/AIDS animals, whereas classic monocytes were greater in SIV + ART animals compared with SIV/AIDS animals. The mean fluorescent intensity of CD163 on intermediate CD14⁺CD16⁺ monocytes was significantly lower for SIV + ART animals compared with SIV/AIDS animals (peripheral blood monocytes and expression; *P* < 0.01) (Table 3).

IENFD and histologic analysis of immune cells were used to compare changes to peripheral neuron afferents and inflammation of the DRG in SIV infection. In terminal SIV/AIDS animals, IENFD exhibited a negative percentage change from pre-infection to necropsy, showing loss of IENFD as a result of untreated SIV infection. There was a positive change in IENFD from pre-infection to necropsy in SIV + ART animals, which was significant when compared with terminal SIV/AIDS, showing an improvement in IENFD with intervention of ART (IENFD measurements; *P* < 0.05) (Table 3). Of note, three animals did not show a positive change in IENFD or return to baseline IENFD,

suggesting that ART may not fully ameliorate reduction in IENFD in SIV infection (Supplemental Figure S3). These data suggest similarities to the HIV population, which shows a subset of patients with persistent loss of IENFD and HIV-associated sensory neuropathy. The lumbar DRG of SIV/AIDS and SIV + ART animals were stained for CD68⁺ resident macrophages, CD163⁺ M2-like macrophages, Mac387⁺ M1-like monocytes, BrdU⁺ newly divided monocytes, and CD3⁺ T cells, which were reported as cells per millimeter squared (lumbar DRG immune cell counts) (Table 3). CD68⁺ resident macrophages and CD3⁺ T cells did not significantly differ between the two groups. CD163⁺ macrophages were significantly lower in SIV + ART animals compared with SIV/AIDS animals (lumbar DRG immune cell counts; *P* < 0.05) (Table 3). Mac387⁺ monocytes and BrdU⁺ monocytes were also significantly lower in SIV + ART animals than SIV/AIDS animals (lumbar DRG immune cell counts; *P* < 0.001 and *P* < 0.05, respectively) (Table 3). Together, these data suggest ART reduces general inflammation seen in high viremia of SIV infection.

Histologic Classification of DRG Neurons by Immunoreactivity for NF200, TrkA, and IB4 in a Rhesus Macaque Model

Neuronal classes were identified as NF200⁺ neurons, IB4⁺ nonpeptidergic neurons, and TrkA⁺ peptidergic neurons

(Supplemental Figure S4, A–C). More important, previous studies have shown overlap between the NF200 and IB4 population of neurons; however, for our comparisons, they will be referred to as separate populations, with the NF200 population representing myelinate neurons and the IB4 population representing nonpeptidergic neurons.^{45,46} The average neuronal diameter of the three populations was compared in uninfected animals, and values significantly differed from one another (analysis of variance < 0.01) (Supplemental Figure S4D). The median diameter of NF200⁺ neurons was 51.28 μm (IQR = 8.20 μm); IB4⁺ neurons, 39.49 μm (IQR = 3.70 μm); and TrkA⁺ neurons, 50.53 μm (IQR = 4.24 μm). As expected and in agreement

with previous reports, TrkA⁺ and IB4⁺ neurons tended to be smaller diameter neurons compared with NF200⁺ neurons.^{8,11,12}

Nonpeptidergic and Peptidergic Neurons Atrophy with SIV Infection

Cell body diameter was used to infer atrophy of NF200⁺, IB4⁺, and TrkA⁺ neurons in SIV infection. All positively stained neurons in the DRG were measured for the largest point-to-point distance of the cell body. The average diameter of NF200⁺ neurons was not significantly different between uninfected, terminal SIV/AIDS, and SIV-infected,

Figure 1 Nonpeptidergic and peptidergic nociceptive neurons atrophy in SIV infection. Neuronal diameters were measured as the longest point-to-point distance of the neuronal cell body of NF200⁺, IB4⁺, and TrkA⁺ neurons and compared between uninfected (SIV–; A01 to A08), terminal SIV/AIDS animals (SIV/AIDS; A12 to A22), and SIV-infected, antiretroviral therapy (ART)-treated animals (SIV + ART; A23 to A28) (Kruskal-Wallis test; Dunn multiple comparison post-hoc analysis). **A:** The average NF200⁺ neuronal diameter did not differ between uninfected, terminal SIV/AIDS, or SIV + ART animals (analysis of variance). **C:** The average IB4⁺ neuronal diameter significantly differed between uninfected, terminal SIV/AIDS, and SIV + ART animals (analysis of variance; $P < 0.01$). Terminal SIV/AIDS animals had smaller cell body diameter compared with uninfected and SIV + ART animals (Dunn post-hoc test; $P < 0.01$ and $P < 0.05$, respectively). **E:** The average TrkA⁺ neuronal diameter significantly differed between uninfected, terminal SIV/AIDS, and SIV + ART animals (analysis of variance; $P < 0.001$), with terminal SIV/AIDS and SIV + ART animals having smaller diameters compared with uninfected animals (Dunn post-hoc test; $P < 0.01$ and $P < 0.01$, respectively). The frequency distributions were graphed and compared between uninfected and SIV-infected animals (two-way analysis of variance; Bonferroni multiple comparison post-hoc analysis). **B:** NF200⁺ neuronal diameter frequencies did not differ between uninfected and SIV/AIDS animals (two-way analysis of variance). **D:** IB4⁺ neuronal diameter frequencies significantly differed between uninfected and SIV/AIDS animals, with Bonferroni post-hoc significance of 5- μm increments denoted on graph (two-way analysis of variance; $P < 0.0001$). **F:** TrkA⁺ neuronal diameter frequencies significantly differed between uninfected and SIV/AIDS animals, with Bonferroni post-hoc significance of 5- μm increments denoted on graph (analysis of variance; $P < 0.0001$). Post-hoc significance (Dunn or Bonferroni multiple comparison) was reported on graphs. Graphs represent the median value of each group with the interquartile range (**A**, **C**, and **E**). $n = 9$ SIV– (**A**–**F**); $n = 10$ SIV/AIDS (**A**–**F**); $n = 6$ SIV + ART (**A**–**F**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

ART-treated animals (Figure 1A). The average diameter of IB4⁺ neurons significantly differed between uninfected, terminal SIV/AIDS, and SIV-infected, ART-treated animals (analysis of variance; $P < 0.01$) (Figure 1C), with significantly larger neuronal diameter for uninfected animals compared with terminal SIV/AIDS animals (post-hoc Dunn test; $P < 0.01$) and significantly larger neuronal diameter for SIV-infected, ART-treated animals compared with terminal SIV/AIDS (post-hoc Dunn test; $P < 0.05$). TrkA⁺ neuronal diameter also significantly differed between the three groups (analysis of variance; $P < 0.001$) (Figure 1E), with significantly smaller diameters in terminal SIV/AIDS and SIV-infected, ART-treated animals compared with uninfected animals (post-hoc Dunn test; $P < 0.01$ and $P < 0.01$, respectively). The frequency of neuronal diameters was segregated by 5- μ m increments and used to identify global changes in neuronal diameter. Frequency distributions were compared between uninfected and terminal SIV/AIDS animals. The frequency distributions of neuronal diameters of NF200⁺ neurons for uninfected and terminal SIV/AIDS did not significantly differ (two-way analysis of variance) (Figure 1B). The frequency distributions of IB4⁺ neuron diameters (two-way analysis of variance; interaction $P < 0.0001$; Bonferroni post-hoc analysis) (Figure 1D) and TrkA⁺ neuron diameters (two-way analysis of variance; interaction $P < 0.0001$; Bonferroni post-hoc analysis) (Figure 1F) significantly differed between uninfected and terminal SIV/AIDS animals. The neuronal diameter was correlated to the viral load in terminal SIV/AIDS animals and SIV + ART animals, inferring changes associated with reduction of SIV viremia. The NF200⁺ and TrkA⁺ neuronal diameter did not correlate with the plasma viral load, whereas the IB4⁺ neuronal diameter was negatively

correlated to the plasma viral load (Supplemental Figure S5) [Spearman correlation; $P > 0.05$ (Supplemental Figure S5A); $P = 0.01$, $R = -0.63$ (Supplemental Figure S5B); $P > 0.05$ (Supplemental Figure S5C)]. These data show no significant difference in neuronal body size of NF200⁺ neurons of uninfected and terminal SIV/AIDS animals. Conversely, IB4⁺ and TrkA⁺ neurons showed population-wide shift in the frequencies of neuronal diameters to a greater prevalence of smaller diameter in animals with SIV infection. In addition, reduction of viral load correlated to a larger diameter in the IB4⁺ neuronal population, suggesting an improved neuronal health with reduced viral load, but TrkA⁺ neuronal diameter remained smaller even with reduction in viral load.

Nonpeptidergic and Peptidergic Neurons Are Selectively Lost in SIV Infection

The percentages of NF200⁺, IB4⁺, and TrkA⁺ neurons were compared among uninfected, SIV/DPI21, terminal SIV/AIDS, and SIV + ART animals. There was no observed difference in the percentage of NF200⁺ neurons between the four groups (Figure 2A). The percentage of IB4⁺ neurons significantly differed among uninfected, SIV/DPI21, terminal SIV/AIDS, and SIV + ART animals (analysis of variance < 0.01) (Figure 2B), with a significantly lower percentage in SIV/AIDS animals compared with uninfected animals (post-hoc Dunn test; $P < 0.01$). The percentage of TrkA⁺ neurons significantly differed between uninfected, SIV/DPI21, terminal SIV/AIDS, and SIV + ART animals (analysis of variance < 0.01) (Figure 2C). The percentage of TrkA⁺ neurons was significantly less in SIV + ART animals compared with

Figure 2 Loss of nonpeptidergic and peptidergic nociceptive neurons with SIV infection. Neuronal populations were quantified and reported as percentage positive neurons in uninfected (SIV-; A01 to A08), early SIV-infected (SIV/DPI21; A09 to A11), terminal SIV/AIDS (SIV/AIDS; A12 to A22), and SIV + antiretroviral therapy (ART) animals (SIV + ART; A23 to A28) (Kruskal-Wallis test; Dunn multiple comparison post-hoc test). **A:** The percentage of NF200⁺ neurons did not significantly differ between uninfected, early SIV-infected, terminal SIV/AIDS, and SIV + ART animals (analysis of variance). **B:** The percentage of IB4⁺ neurons significantly differed between uninfected, early SIV infection, terminal SIV/AIDS, and SIV + ART animals (analysis of variance; $P < 0.01$), with a significantly lower percentage of IB4⁺ neurons in terminal SIV/AIDS compared with uninfected animals (Dunn post-hoc test; $P < 0.01$). **C:** The percentage of TrkA⁺ neurons significantly differed between uninfected, early SIV infection, terminal SIV/AIDS, and SIV + ART animals (analysis of variance; $P < 0.01$). The percentage of TrkA⁺ neurons was significantly less in SIV + ART compared with uninfected animals (Dunn post-hoc test; $P < 0.05$), and significantly less in terminal SIV/AIDS and SIV + ART compared with SIV/days post infection (DPI) 21 animals (Dunn post-hoc test; $P < 0.05$ and $P < 0.05$, respectively). Post-hoc significance (Dunn multiple comparison) was reported. Graphs represent the median value of each group with the interquartile range (A–C). $n = 9$ SIV- (A–C); $n = 3$ SIV/DPI21 (A–C); $n = 10$ SIV/AIDS (A–C); $n = 6$ SIV + ART (A–C). * $P < 0.05$, ** $P < 0.01$.

uninfected animals (post-hoc Dunn test; $P < 0.05$). Terminal SIV/AIDS and SIV + ART animals showed a significantly lower percentage of TrkA⁺ neurons compared with SIV/DPI21 animals (post-hoc Dunn test; $P < 0.05$ and $P < 0.05$, respectively).

Cleaved Caspase 3 Accumulates in DRG Neuronal Cell Bodies in SIV Infection

Caspase 3 cleavage traditionally signifies a late apoptotic event and was used as a marker of end-stage sensory neuron apoptotic signaling. Relative caspase 3 mRNA was determined through RNAscope, and there was no observed difference in caspase 3 mRNA expression in DRG neurons of uninfected, terminal SIV/AIDS, and SIV + ART animals (data not shown), suggesting that positive staining for cleaved caspase 3 protein was a result of increased proteolytic activation without increased caspase 3 mRNA expression. Cleaved caspase 3 protein was visualized as granular staining in the cytosol of neuronal cell bodies (Figure 3, A–D), and the percentage of cleaved caspase 3⁺ neurons was significantly different among uninfected, terminal SIV/AIDS, and SIV + ART animals (analysis of

variance < 0.001 ; SIV/DPI21 not included in statistical analysis because $n = 2$) (Figure 3E). Cleaved caspase 3⁺ neurons were significantly greater in SIV/AIDS animals compared with uninfected (post-hoc Dunn test; $P < 0.01$) and SIV + ART animals (post-hoc Dunn test; $P < 0.05$). There was no significant difference between uninfected and SIV + ART animals. The percentage of cleaved caspase 3⁺ neurons was correlated to the average neuronal diameter (atrophy) in uninfected and terminal SIV/AIDS animals, to infer a correlation between atrophy and apoptotic events. There was no significant correlation between cleaved caspase 3⁺ neurons and the average diameter of NF200⁺ neurons (Spearman correlation; $P > 0.05$) (Figure 3F). There was a significant negative correlation between the percentage of cleaved caspase 3⁺ neurons and the average diameter of IB4⁺ neurons (Spearman correlation; $P < 0.01$, $R = -0.70$) (Figure 3G) and TrkA⁺ neurons (Spearman correlation; $P < 0.001$, $R = -0.72$) (Figure 3H). Of note, cleaved caspase 3 was also visualized in the axons of DRG neurons; however, DRG axons in tissue sections are indeterminate of innervation and could be innervating peripheral targets or the dorsal horn of the spinal column. Immunofluorescence for

Figure 3 Accumulation of cytosolic cleaved caspase 3 in dorsal root ganglia (DRG) neurons with SIV infection. Cleaved caspase 3 protein (activated caspase 3) was visualized in DRG neuron cytosol and compared between uninfected (SIV–; A01 to A04 and A08), early SIV-infected (SIV/DPI21; A09 and A10), terminal SIV/AIDS (SIV/AIDS; A12 and A14 to A22), and SIV + antiretroviral therapy (ART) animals (SIV + ART; A23 to A24 and A26 to A28) (Kruskal-Wallis test; Dunn multiple comparison post-hoc test; SIV + DPI21 not included in analysis). **A–D:** Representative images show cytosolic granules of cleaved caspase 3 in DRG neurons (arrows). **E:** The percentage of cleaved caspase 3⁺ neurons significantly differed between uninfected, terminals SIV/AIDS, and SIV + ART animals (analysis of variance; $P < 0.001$), with significantly greater cleaved caspase 3⁺ neurons in SIV/AIDS compared with uninfected and SIV + ART animals (Dunn post-hoc test; $P < 0.01$ and $P < 0.05$, respectively). The average neuronal cell body diameter of uninfected and terminal SIV/AIDS animals (denoting atrophy) was compared with the percentage of cleaved caspase 3⁺ neurons (Spearman correlation). **F:** The diameter of NF200⁺ neurons did not significantly correlate to the percentage of cleaved caspase 3⁺ neurons. **G and H:** The percentage of cleaved caspase 3⁺ neurons was significantly, negatively correlated to the average IB4⁺ neuronal diameter ($P < 0.01$, $R = -0.70$; **G**) and the average TrkA⁺ neuronal diameter ($P < 0.001$, $R = -0.72$; **H**). Post-hoc significance (Dunn multiple comparison) was reported on graphs. If Spearman correlation was significant, a linear regression (black lines) was added to graph for representation of correlational trend. †SIV/DPI21 was not included in statistical analysis because $n = 2$. Graphs represent the median value of each group with the interquartile range (**E**). $n = 5$ SIV– (**A–H**); $n = 2$ SIV/DPI21 (**A–H**); $n = 9$ SIV/AIDS (**A–H**); $n = 5$ SIV + ART (**A–H**). * $P < 0.05$, ** $P < 0.01$. Scale bars = 10 μm (**A–D**). Original magnification, $\times 100$ (**A–D**). DPI, days post infection.

Figure 4 Cleaved caspase 3 colocalizes predominantly with nonpeptidergic sensory neurons. Immunofluorescence for cleaved caspase 3, NF200, IB4, and TrkA was used to identify the class of neurons with cytosolic cleaved caspase 3. Representative images of uninfected animals (SIV[−]) and terminal SIV/AIDS (SIV/AIDS) show cleaved caspase 3 (green; **A**), TrkA⁺ neurons (red; **B**), IB4⁺ neurons (far red; **C**), and merged (**D**). In SIV/AIDS merged representative images, cleaved caspase 3⁺ nonpeptidergic neurons are denoted with **arrows**. Representative images of SIV[−] and SIV/AIDS show cleaved caspase 3 (green; **E**), NF200⁺ neurons (red; **F**), and merged (**G**). In SIV/AIDS merged representative images, cleaved caspase 3⁺ and NF200⁺ neurons are denoted with **arrows**. Scale bars = 20 μm (**A–G**). Original magnification, $\times 60$ (**A–G**).

cleaved caspase 3, NF200, IB4, and TrkA was used to determine the colocalization of caspase 3 cascade activation in different classes of neurons in SIV-infected animals (Figure 4). Uninfected animals ($n = 3$) showed no positive staining for cleaved caspase 3 in the cell body of NF200⁺, IB4⁺, or TrkA⁺ neurons (denoted SIV[−]) (Figure 4). Terminal SIV/AIDS animals showed a median of 9.74% (IQR = 0.13%; $n = 2$) of NF200⁺ neurons colocalized with cleaved caspase 3, 49.44% (IQR = 9.43%; $n = 3$) of IB4⁺ neurons colocalized with cleaved caspase 3, and 8.97% (IQR = 3.17%; $n = 3$) of TrkA⁺ neurons colocalized with cleaved caspase 3 (denoted SIV/AIDS) (Figure 4). Interestingly, IB4⁺ nonpeptidergic neurons showed a significant degree of overlap with cleaved caspase 3⁺ sensory neurons, but NF200⁺ or TrkA⁺ sensory neurons showed less cleaved caspase 3 staining in the cell body. These data suggest that caspase 3 cascade activation

is occurring largely in nonpeptidergic neurons with progression to terminal SIV/AIDS.

ATF3 Accumulates in the Nucleus of Sensory Neurons during SIV Infection

ATF3 is an integral response element for axon terminal regeneration in sensory neurons and was observed in the nucleus of DRG neurons (Figure 5, A–D). The percentage of neurons with ATF3⁺ nuclei was significantly different among uninfected, terminal SIV/AIDS, and SIV + ART animals (analysis of variance < 0.01 ; SIV/DPI21 was not included in statistical analysis because $n = 2$) (Figure 5E). The percentage of ATF3⁺ nuclei was significantly greater in terminal SIV/AIDS animals compared with uninfected animals ($P < 0.01$; post-hoc test). There was no significant difference between SIV + ART animals and uninfected or

Figure 5 Increased nuclear activating transcription factor 3 (ATF3) in SIV infection. ATF3 was visualized in dorsal root ganglia neuron nucleus and compared between uninfected (SIV–; A01 to A04 and A06 to A08), early SIV-infected (SIV/DPI21; A09 and A10), terminal SIV/AIDS (SIV/AIDS; A12 and A14 to A22), and SIV + ART animals (SIV + ART; A23, A24, and A26 to A28) (Kruskal-Wallis test; Dunn multiple comparison post-hoc test; SIV + DPI21 not included in analysis). **A–D:** Representative images show dense ATF3 immunoreactivity in the nucleus of positive neurons (arrows). **E:** The percentage of neurons with ATF3⁺ nuclei was significantly different between uninfected, terminal SIV/AIDS, and SIV + ART animals (analysis of variance; $P < 0.01$), with a significantly greater percentage of ATF3⁺ neurons in SIV/AIDS animals compared with uninfected animals (Dunn post-hoc test; $P < 0.01$). Post-hoc significance (Dunn multiple comparison) was reported on graphs. [†]SIV/DPI21 was not included in statistical analysis because $n = 2$. Graph represents the median value of each group with the interquartile range (**E**). $n = 7$ SIV– (**A–E**); $n = 2$ SIV/DPI21 (**A–E**); $n = 9$ SIV/AIDS (**A–E**); $n = 5$ SIV + ART (**A–E**). $**P < 0.01$. Scale bar = 50 μ m (**A–D**). Original magnification, $\times 40$ (**A–D**). ART, antiretroviral therapy; DPI, days post-infection.

terminal SIV/AIDS animals. Immunofluorescence for ATF3, NF200, IB4, and TrkA was used to determine the class of ATF3-expressing neurons in SIV-infected animals (Figure 6). Uninfected animals ($n = 3$) showed no positive staining for ATF3 in the neuronal nuclei of NF200⁺, IB4⁺, or TrkA⁺ neurons (denoted SIV–) (Figure 6). Terminal SIV/AIDS animals showed a median of 3.43% (IQR = 0.12%; $n = 2$) of NF200⁺ neuronal nuclei positive for ATF3, 7.07% (IQR = 1.66%; $n = 3$) of IB4⁺ neuronal nuclei positive for ATF3, and 10.22% (IQR = 8.99%; $n = 3$) of TrkA⁺ neuronal nuclei positive

for ATF3 (denoted SIV/AIDS) (Figure 6). Most ATF3⁺ neurons were found to be either IB4⁺ nonpeptidergic neurons or TrkA⁺ peptidergic neurons in terminal SIV/AIDS animals, with minimal overlap with NF200⁺ neurons.

Discussion

Herein, we characterized damage to peripheral sensory neurons in an accelerated model of HIV infection with or without ART. Neuronal populations were classified as NF200⁺ myelinated neurons, IB4⁺ nonpeptidergic nociceptors, or TrkA⁺ peptidergic nociceptors and assessed for neuronal atrophy, regeneration signaling, and activation of apoptosis in uninfected animals, early and terminal SIV-infected animals, and SIV-infected animals receiving a clinically relevant ART regimen. Successful reduction of viral load by ART dramatically reduced SIV-associated immune activation and DRG pathologic changes (viral load was not suppressed to undetectable). In the presence of ART, plasma inflammatory marker sCD14, sCD163, CCL2, and CCL5; monocyte expansion (percentage monocytes); inflammatory monocyte phenotypic shift (percentage CD14⁺CD16⁺, percentage CD14[–]CD16⁺, and CD163 mean fluorescent intensity); and myeloid infiltration of the DRG were all significantly lower, compared with untreated SIV infection. Over the course of infection, there was a significant improvement of IENFD, but three of the SIV + ART animals did not fully recover to baseline IENFD measurements. Taken together, ART may not fully remedy loss IENFD over the course of SIV infection.

As HIV-DSP presents with symmetric nociceptive pain, hypersensitivity to temperatures, and numbness or tingling in the extremities, we aimed to investigate disruption of nonpeptidergic and peptidergic neuron function and assessed the extent of sensory neuron damage in SIV infection. Using neuronal diameter as an indicator of atrophy and surrogate for neuronal dysfunction, a marked decline was observed in the average diameter of nonpeptidergic neurons in SIV infection; however, SIV-infected animals receiving ART had greater cell body diameters of nonpeptidergic neurons than terminal SIV/AIDS animals, showing a protective effect of viral suppression. Conversely, in the peptidergic neurons, significant atrophy was observed in both untreated and ART-treated, SIV-infected animal groups compared with uninfected control animals. Through comparison of total population diameter frequencies, nonpeptidergic and peptidergic neurons showed a population-wide shift in cell body size to that of a smaller diameter in terminal SIV/AIDS animals compared with uninfected control animals. In addition, comparison of the percentage of nonpeptidergic and peptidergic neurons between uninfected animals, SIV-infected animals, and SIV-infected animals receiving ART showed a selective loss of nociceptive neurons, with NF200⁺ neurons being unaffected. More

Figure 6 Activating transcription factor 3 (ATF3) localizes with nonpeptidergic and peptidergic neurons. Immunofluorescence for ATF3, NF200, IB4, and TrkA was used to identify the class of neurons with nuclear localization of ATF3. Representative images of uninfected animals (SIV–) and terminal SIV/AIDS (SIV/AIDS) show ATF3⁺ nuclei (green; **A**), TrkA⁺ neurons (red; **B**), IB4⁺ neurons (far red; **C**), and merged (**D**). In SIV/AIDS merged representative images, ATF3⁺ nonpeptidergic and peptidergic neurons are denoted with **arrows**. Representative images of SIV– and SIV/AIDS show ATF3⁺ nuclei (green; **E**), NF200⁺ neurons (red; **F**), and merged (**G**). In SIV/AIDS merged representative images, ATF3⁺ and NF200⁺ neurons are denoted with **arrows**. Scale bar = 50 μ m (**A–G**). Original magnification, $\times 20$ (**A–G**).

important, even with ART, peptidergic neurons remained significantly lower compared with uninfected and SIV/DPI21 animals. Taken together, SIV infection is detrimental to nociceptive neurons and generates an unfavorable environment for both nonpeptidergic and peptidergic neurons. Intervention with ART may improve the pathologic changes observed in nonpeptidergic neurons; however, even with reduction in viremia and inflammation, peptidergic neurons seemed susceptible to atrophy, damage, and death.

Cleavage of caspase 3, an important effector caspase and traditional late-stage marker of apoptosis, was observed to different degrees in peripheral neurons in SIV infection. Caspase 3 is expressed in mature olfactory neurons throughout the cell bodies, axons, and synaptic terminals.⁴⁷ On a damage event to sensory neurons, caspase 3 cleavage is initiated at the site of injury and, in the case of axotomy, procaspase 3 expression is significantly increased.^{47,48} No

change was observed in caspase 3 expression in DRG neurons in SIV infection, suggesting increase in caspase 3 was the result of proteolytic processing. An increased number of cleaved caspase 3⁺ neurons was observed in early SIV infection and was greater at terminal SIV/AIDS. Intervention with ART resulted in a lower number of cleaved caspase 3⁺ neurons compared with untreated SIV infection. Interestingly, a significant portion of the IB4⁺ nonpeptidergic neurons (49.44% of IB4⁺ neurons) colocalized with cleaved caspase 3 staining in SIV-infected animals, whereas NF200⁺ and TrkA⁺ neurons colocalized to a lesser extent (9.74% and 8.97%, respectively). These data suggest that caspase 3-mediated mechanisms of apoptosis may be more pronounced in the IB4 nonpeptidergic neurons. In this study, a characteristic marker of late-stage apoptosis was used, but, other mechanisms of cell death, such as autophagy or necrosis, were not investigated,

which may be more pertinent to TrkA⁺ peptidergic neurons. Although caspase 3 was used to infer apoptotic signaling, peripheral neurons in a model of long-term experimental diabetic neuropathy showed accumulation of cleaved caspase 3 in the cell body, but the increased caspase 3 activation was not associated with cell death,⁴⁹ suggesting a possible nonapoptotic mechanism of caspase 3 in sensory neurons. Cleaved caspase 3 was quantified in the neuronal cell body, but the initial caspase activation may not have occurred in the cell body, as cleaved caspase 3 was also observed in the axons of peripheral neurons. Further studies are needed to address the source of caspase 3 activation in peripheral neurons during SIV infection, whether it be at the cell body or axon, to identify possible nonapoptotic roles of caspase 3, to determine a threshold for apoptosis in sensory neurons, and to address contributions of other cell death mechanisms.

Peripheral sensory neurons have both a dynamic and direct interaction with body, making them particularly susceptible to changes in disease states, and in HIV-DSP, patients present with significant and progressive loss of IENFD. We investigated expression changes in ATF3, an integral transcription factor of the regeneration program in sensory neurons. In SIV infection, there was a greater number of DRG neurons with ATF3 localized to the nucleus, suggesting an induction of regeneration-associated gene transcripts. Although this is a cross-sectional study, ATF3 accumulation may be transient over the course of infection. In addition, neurons with high degree of nuclear ATF3 were identified to be mostly IB4⁺ nonpeptidergic or TrkA⁺ peptidergic neurons (7.07% and 10.22%, respectively), with NF200⁺ neurons colocalizing to a lesser extent (3.43%). These findings support the notion that loss of IENFs of sensory neurons may promote regenerative signaling in nonpeptidergic and peptidergic neurons. Although we looked at a specific transcription factor, further studies are needed to identify synergism with other transcription factors and the involvement of specific pro-regenerative proteins and transcripts in the presence of SIV infection.

In summary, SIV infection generates an environment that drives neuropathologic changes in the DRG, with a class-specific impact on nonpeptidergic and peptidergic neurons. Loss of nonpeptidergic neurons in SIV infection was lessened by intervention with ART; however, peptidergic neurons appeared particularly susceptible to cell death and atrophy, in both the absence and the presence of ART. Future investigations should determine and compare resilience of nonpeptidergic and peptidergic neurons in different pathophysiological conditions, such as axotomy, inflammation, and infection. Although reduction was observed in cleaved caspase 3 and ATF3 with viral load reduction, subtler changes in nonpeptidergic and peptidergic neurons, such as expression of channel proteins, neuropeptides, and cytokines, were not studied. Further studies are needed to address long-term changes in nonpeptidergic and

peptidergic neurons in the context of successful viral suppression and chronic, low-grade inflammation, giving insight into mechanisms of hypersensitivity, damage to peripheral axon terminals, and class-specific vulnerability to changes during HIV infection.

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Author Contributions

This project was designed and conceived by T.H.B; data were acquired and analyzed by J.A.R., G.G., R.W., M.D.S., and J.R.L; pathology was assessed by A.D.M.; statistical analysis and interpretation were performed by J.A.R., G.G., R.W., J.R.L., M.S., and T.H.B; the manuscript was drafted by J.A.R. and T.H.B and critical revisions performed by J.A.R., A.D.M., and T.H.B.

Supplemental Data

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