IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Neuroinflammation in the Dorsal Root Ganglia and Dorsal Horn Contributes to Persistence of Nociceptor Sensitization in SIV-Infected Antiretroviral Therapy-Treated Macaques

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HIV-associated distal sensory polyneuropathy (HIV-DSP) is a significant contributor of neuropathic pain in people with HIV.1,2 With the introduction of antiretroviral therapy (ART), the incidence of HIV-DSP has decreased from an estimated 30% to 50% to approximately 25% of people with HIV.2 However, this estimation fails to correctly account for the increased incidence of HIV-DSP in demographic populations with low ART adherence (54% to 83%).1,3,4 In the post-ART era, HIV-DSP is likely due to chronic HIV infection and subsequent inflammation, independent of viral load and CD4 cell count.5 Clinically, HIV-DSP presents as severe pain, hyperalgesia, and loss of sensation in the distal limbs.5 Its pathophysiology is marked by peripheral nerve fibers degeneration, immune cell infiltration to the dorsal root ganglia (DRG), and direct damage to DRG neurons.6–8 Simian immunodeficiency virus (SIV)—infected rhesus macaques are used to model the characteristics and

Despite the development of antiretroviral therapy (ART), HIV-associated distal sensory polyneuropathy remains prevalent. Using SIV-infected rhesus macaques, this study examined molecular mechanisms of peripheral and central sensitization to infer chronic pain from HIV infection. Previous studies identified atrophy in nociceptive neurons during SIV infection, which was associated with monocyte infiltration into the dorsal root ganglia (DRG). However, the sensory signaling mechanism connecting this pathology to symptoms remains unclear, especially because pain persists after resolution of high viremia and inflammation with ART. We hypothesized that residual DRG and dorsal horn neuroinflammation contributes to nociceptive sensitization. Using three cohorts of macaques [uninfected (SIV−), SIV-infected (SIV+), and SIV infected with ART (SIV+/ART)], this study showed an increase in the cellular and cytokine inflammatory profiles in the DRG of SIV+/ART macaques compared with uninfected animals. It found significant increase in the expression of nociceptive ion channels, TRPV1, and TRPA1 among DRG neurons in SIV+/ART compared with uninfected animals. SIV-infected and SIV+/ART animals showed reduced innervation of the nonpeptidergic nociceptors into the dorsal horn compared with uninfected animals. Finally, there were a significantly higher number of CD68+ cells in the dorsal horn of SIV+/ART macaques compared with uninfected animals. In summary, these data demonstrate that neuroinflammation, characteristics of nociceptor sensitization, and central terminal atrophy persists in SIV+/ART animals. (Am J Pathol 2023, 193: 2017–2030; https://doi.org/10.1016/j.ajpath.2023.08.014)
underlying mechanisms of HIV-DSP. As in people with HIV, SIV infection of rhesus macaques leads to atrophy of nociceptor nerve fibers as a consequence of monocyte/macrophage-mediated damage to the DRG. Mono-nuclear cell infiltration into the DRG is strongly associated with pathological changes, which are not entirely resolved with ART. Within the DRG, the C-fiber nociceptors contribute to slow enduring pain and temperature sensations described with HIV-DSP. The C-fiber nociceptors are divided into two subclasses: tropomyosin receptor kinase A–expressing (TRKA+) peptidergic nociceptors and isolectin B4–expressing (IB4+), nonpeptidergic nociceptors. Atrophy of both TRKA+ and IB4+ nociceptors occurs in the DRG of SIV-infected macaques. With ART only, IB4+ nociceptors are spared from pathological damage. These observations suggest potential chronic damage to neuronal subsets despite ART, but mechanisms leading to altered nociceptive signaling and dorsal root innervation into the spinal cord are unknown.

Long-term cytokine exposure can contribute to peripheral sensitization of nociceptors through the increased expression of nociceptive ion channels, including transient potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1). Knockout and antagonist studies for TRPV1 and TRPA1 have indicated that these ion channels are essential for the development of inflammatory thermal and mechanical hyperalgesia. Activation of these ion channels can initiate action potentials in primary nociceptors, whereas localization to the dorsal horn can further increase calcium permeability and release of synaptic vesicles, leading to central sensitization. Central sensitization develops through peripheral sensory neuron hyperexcitability and neuronal glial interactions in the dorsal horn. It is well described that HIV/SIV infection is associated with activation of central nervous system (CNS) microglia and mononuclear cell trafficking into the spinal cord. Despite this, the characterization of the tissue damage following immunological resolution has yet to be described in the context of HIV-DSP. This study aimed to characterize the following in SIV infection with or without ART: i) the neuroinflammatory burden of mononuclear cells and derived cytokines in the DRG, ii) alterations in gene expression and trafficking of TRPV1 and TRPA1 in afferent nociceptor subtypes, and iii) alterations in nociceptor subtype innervation and mononuclear cell capacity in the dorsal horn.

Materials and Methods

Animal Model

Indian rhesus macaques (Macaca mulatta) were used in this study as a model of HIV infection. NIH notice number NOT-O15-102 recognizes primates as an “acutely scarce resource” and specifically justifies primarily single-sex studies. Animals were housed at either the Tulane National Primate Research Center or the New England Regional Primate Center. Because of differences in availability of DRG and spinal cord tissue, not all animals were used in every experiment (Supplemental Table S1). Animals A01 to A19 serve as uninfected controls (n = 19) (referred to as SIV−). Two SIV− animals were CD8 depleted (A01 and A02), and 16 were not CD8 depleted (A03-A19). Animals A20 to A37 were infected with SIVmac251 viral swarm (5 ng of p27; Tulane National Primate Research Center’s Viral Core) followed by CD8 depletion (Nonhuman Primate Reagent Resource), as previously described. CD8 depletion following SIVmac251 infection results in a highly reproducible SIV rapid progression model with a higher incidence and degree of peripheral nerve pathology than seen in SIV-infected non–CD8-depleted animals mirroring the characteristics of a natural SIV rapid progressor. Animals in CD8 depletion, infection, and ART are available in Supplemental Figure S1.

Hematoxylin and Eosin Staining and Pathological Assessment

Lumbar spinal cord (LSC) sections were prepared and sectioned as previously reported. Sections were deparaffinized then rehydrated and stained with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO) for 8 minutes. Slides were differentiated in 1% acetic acid, blued in ammonia water, counterstained using Eosin Y-solution 0.5% (Sigma-Aldrich) for 3 minutes, dehydrated, and mounted using Vectamount (Vector Laboratories, Newark, CA). Pathological analysis was performed by a board-certified veterinary anatomical pathologist (A.D.M.) blinded to animal conditions. Tissue sections were assessed for whole pathology identifying the severity of SIV myelitis and white matter degeneration (Supplemental Table S1).

Viral Load Measurement

Plasma viral loads were determined as previously described and reported. The threshold sensitivity was 15 copies Eq/mL, with a mean interassay CV <25%.
ELISA

Plasma IL-6 was determined using a Mesoscale Discovery V-plex human inflammatory panel 1, according to manufacturer’s instruction (Mesoscale Discovery, Rockville, MD). Lower limit of detection (LLOD) for V-plex human inflammatory panel was 0.06 pg/mL for IL-6. Plasma IL-1β was determined using the Human IL-1 beta/IL-1F2 Quantikine HS enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). The LLOD was 0.063 pg/mL and the upper limit of detection was 8 pg/mL for IL-1β. Tissue lysates of DRGs from SIV− and SIV+/ART macaques were prepared by homogenization in Pierce radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA). Protein concentration was determined using a Pierce 660 protein assay kit (Thermo Fisher Scientific). Total protein (30 µg) was run per sample using a S-plex human inflammatory panel 1 [interferon (IFN)-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, and tumor necrosis factor (TNF)-α] (Mesoscale Discovery). Samples were spiked with S-plex human inflammatory panel standard for a final spike concentration of 156.46 fg/mL for IL-1β, 44.72 fg/mL for IL-6, 474.61 fg/mL for IL-12p70, 52.92 fg/mL for IL-4, 252.92 fg/mL for IL-17A, and 78.32 fg/mL for IL-2. The concentration of the spike was determined based on the calculated concentration of S-plex standard 6, which both have a recovery range of 85% to 95%. The calculated spike was subtracted from the sample concentration and the femtograms of IL-1β to 95%. The calculated spike was subtracted from the sample concentration and the femtograms of IL-1β to 95%. The calculated spike was subtracted from the sample concentration and the femtograms of IL-1β to 95%. The calculated spike was subtracted from the sample concentration and the femtograms of IL-1β to 95%. The calculated spike was subtracted from the sample concentration and the femtograms of IL-1β to 95%.

Dual RNAscope and Immunofluorescence

All antibodies used are detailed in Table 1. Lumbar DRG and sacral DRG tissue sections were treated with 1× antigen retrieval 6 buffer according to the Opal manual 4-color immunohistochemistry kit protocol (Akoya Biosciences, Marlborough, MA). Tissue was blocked using the Opal blocking buffer (Akoya Biosciences), and hybridization and amplification of the TRPV1 and TRPA1 RNA probes were performed. Target RNA was visualized using horse-radish peroxidase—tagged fluorophore development with Opal dye 570. Once developed, subsequent multiplex immunohistochemistry (IHC) for IB4 (Vector Laboratories) (1:50) and TRKA (Abcam, Cambridge, UK) (1:2000) labeling was performed according to Opal multiplex protocols. Slides were counterstained with DAPI (Akoya Biosciences). Tissue sections were imaged using the Keyence BZ-X700 microscope, with 15 to 20 × 20 magnification nonoverlapping images (for DRG) or whole image stitches (for LSC) taken across the tissue section and used to determine the colocalization of TRPV1 and TRPA1 RNA to IB4+ or TRKA+ nociceptors. The innervation of TRKA and IB4 nociceptors was also determined using stained LSC images in which the area of IB4 or TRKA immuno-reactivity was divided by the total area of lamina 1 and 2.

Table 1  Antibodies Used in Histologic Analysis

<table>
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<th>Target</th>
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<th>Catalog no.</th>
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<td>Isolectin B4</td>
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<td>Vector Laboratories (Newark, CA)</td>
<td>B-1205</td>
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<tr>
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<td>Rabbit</td>
<td>Abcam (Cambridge, UK)</td>
<td>ab76291</td>
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<td>CD68</td>
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<td>Dako Cytomation (Santa Clara, CA)</td>
<td>M0814</td>
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<td>NBP1-71774</td>
<td>1:750</td>
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<tr>
<td>TRPA1</td>
<td>Rabbit</td>
<td>Novus Biologicals</td>
<td>NB110-40763</td>
<td>1:250</td>
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</tbody>
</table>

*Not an antibody [biotinylated *Griffonia simplicifolia* lectin I (GSL I) isolectin B4].

TRKA, tropomyosin receptor kinase A; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient potential vanillolid 1.
Opal multiplex IHC of the LSC was used to visualize the colocalization of TRPV1 and TRPA1 protein to the central terminals of IB4 or TRKA nociceptors. Tissue sections were prepared for staining using a 1× sodium citrate antigen retrieval solution (Vector Laboratories) followed by a serum-free protein block (Dako Laboratories). Blocking was performed using Bloxall (Vector Laboratories). Target protein was visualized through development of the diaminobenzidine substrate chromogen system (Dako Cytomation). Counterstain was performed with hematoxylin (1:10) (Sigma-Aldrich). For analysis of TRPV1+ neurons in the DRG, nonoverlapping sections (8 to 13; magnification ×20) were imaged where TRPV1+ neurons were divided by the total neurons per image. Individual dorsal horns were used as biological replicates. With the Keyence BZ-X700 software version 1.4.1.1, the colocalization of TRPV1 or TRPA1 to either IB4+ or TRKA+ nociceptors was calculated by measuring the direct area of overlap between the two fluorescent channels. These stains were also used for the analysis of IB4 and TRKA nociceptor innervation into the dorsal horn. The area of lamina 1 or 2 was selected as the target area. A threshold was set across images for the fluorescent area of IB4+ or TRKA+ expression within the target area, from which the percentage area of expression was determined. For staining of TRPV1 in DRG and CD68 in DRG and LSC, tissue sections were prepared for staining using a 1× antibody solution (Novus Biologicals). Slides were counterstained with DAPI (Akoya Biosciences) and imaged using the Krypton-argon laser (Keyence BZ-X700 microscope) to capture the entirety of both dorsal horns. Individual dorsal horns were used as biological replicates. With the Keyence BZ-X700 software version 8 (GraphPad Software Inc., Boston, MA). All statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., Boston, MA). All experimental data sets were tested for normality using an Anderson-Darling test (P < 0.05) where comparisons assessed did not follow a gaussian distribution. All tests performed yielded a statistically significant result with a P ≤ 0.05 and a confidence level of 95%. For comparison between two repeated measures as in plasma cytokine levels, the Wilcoxon signed-rank test was used. For comparison between two unpaired measures as in DRG cytokine levels, the U-test was used. For all other comparisons among the SIV−, SIV+, and SIV+/ART animals, unpaired groups Kruskal-Wallis one-way analysis of variance was used. If found significant, a post hoc Dunn’s multiple comparison test was performed to identify significant differences within the 3 groups.

Results

Soluble and Cellular Markers of Mononuclear Inflammation Remain Elevated in the Plasma and DRGs of SIV+/ART Macaques

Because IL-1β and IL-6 are upstream regulators of TRPV1/TRPA1 transcription, they were measured in both plasma (Table 2) and DRG lysates (Figure 1). Plasma IL-6 was significantly increased from preinfection to necropsy in the SIV+ (Wilcoxon signed-rank, P < 0.001; Table 2) and in SIV+/ART animals (Wilcoxon signed-rank, P < 0.05; Table 2). There was no significant difference in plasma IL-1β from preinfection to necropsy in either of the groups (Table 2). Consistent with previous reports, there was a significant difference among groups in the number of CD68+ cells in the DRG (Kruskal-Wallis, P < 0.01, H = 9.760; Figure 1A). Specifically, CD68+ cells were significantly increased in SIV+ compared with SIV− and SIV+/ART (Dunn’s multiple comparison, P < 0.05 for both; Figure 1A). Because cytokines can interact with primary nociceptors sensitizing them to peripheral stimuli, the cytokine burden (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, and TNF-α) in the DRGs was determined. A significant increase in IL-1β (U-test, P < 0.05; Figure 1B), but not IL-6, was found in the DRGs of SIV+/ART macaques compared with uninfected controls. There were no significant differences in DRG concentration of IL-2, IL-17A, IL-12p70, and IL-4 between SIV− and SIV+/ART groups (Supplemental Figure S2). Concentrations of IFN-γ, IL-10, and TNF-α in the DRG were below the LLOD. To assess presence of viral replication in the DRG, RNAscope was used to determine the number of SIVmace239+ cells in the DRGs of SIV+ and SIV+/ART animals. There were no significant differences in the number of SIV RNA+ cells between SIV+ and SIV+/ART animals (Supplemental Figure S3). These results are consistent with the hypothesis that inflammation and viral reservoirs remain in the DRG despite plasma viral suppression.
Expression of TRPV1 and TRPA1 is Increased in the DRGs of SIV+/ART Macaques

Because IL-1β can drive expression of nociceptive ion channels such as TRPV1 and TRPA1, the study next determined transcriptional changes of TRPV1 and TRPA1 in the DRG. Using RNASequencing in situ hybridization, the study examined TRPV1 and TRPA1 RNA in DRG neurons among SIV−, SIV+ and SIV+/ART groups (Figure 2, A and C). The percentage of TRPV1-RNA+ neurons was significantly different among SIV−, SIV+, and SIV+/ART groups (Kruskal-Wallis test, P < 0.01, H = 12.17; Figure 2B). The percentage of TRPV1-RNA+ neurons in the DRG was significantly greater in SIV+/ART compared with that in both SIV+ and SIV− (Dunn’s multiple comparison, P < 0.01 for both; Figure 2B). The percentage of TRPA1-RNA+ neurons was significantly different among SIV−, SIV+, and SIV+/ART groups (Kruskal-Wallis test, P < 0.01, H = 9.905; Figure 2D). Specifically, the percentage of TRPA1-RNA+ neurons in the DRG was significantly greater in SIV+/ART compared with that in the SIV− animals (Dunn’s multiple comparison, P < 0.01; Figure 2D). No significant differences were seen in gene expression of either TRPV1 or TRPA1 in SIV+ animals compared with that in SIV− animals. To assess whether TRPV1 protein expression mirrors change seen in TRPV1 RNA expression across animal groups, the study determined the percentage of TRPV1 protein+ neurons in the DRG. The median (IQR) percentage of TRPV1− protein+ neurons in the DRGs of animal groups is as follows: SIV−, 22.32% (16.97%−24.96%) (n = 6); SIV+, 22.46% (8.37%−31.22%) (n = 10); and SIV+/ART, 32.60% (31.53%−35.04%) (n = 6). The percentage of TRPV1-protein+ neurons was significantly different among SIV−, SIV+, and SIV+/ART groups (Kruskal-Wallis, P < 0.05, H = 7.991; Supplemental Figure S4). Specifically, the percentage of TRPV1-protein+ neurons in the DRG was significantly greater in SIV+/ART compared with SIV+ and SIV− animals (Dunn’s multiple comparison, P < 0.05 for both; Supplemental Figure S4). The analysis of TRPV1 protein expression among DRG neurons confirmed an overall relative increase in nociceptive ion...
channel transcription and translation in specifically SIV+/ART animals. This finding suggests that the increase in nociceptive ion channel expression is independent of high viremia and severe DRG pathology.

The neuronal subtype contributing to an increase in TRPV1+ and TRPA1+ neurons was determined by dual RNAscope and immunofluorescence. Colocalization of RNA transcripts to TRKA+ and IB4+ nociceptors was compared among the SIV−, SIV+, and SIV+/ART animals (Figure 3A). There were significant differences in the percentage of TRPV1-expressing IB4+ neurons (Kruskal-Wallis, $P < 0.05$, $H = 7.563$; Figure 3B) and TRPV1-expressing TRKA+ neurons among SIV−, SIV+, and SIV+/ART animals (Kruskal-Wallis, $P < 0.05$, $H = 6.532$).

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**Figure 1** The number of CD68+ macrophages and IL-1β concentration in the dorsal root ganglion (DRG) remain elevated in simian immunodeficiency virus (SIV)−infected with antiretroviral therapy (SIV+/ART) macaques. A: CD68+ cells were visualized through immunohistochemistry and calculated by division of image area for SIV uninfected (SIV−), SIV-infected (SIV+), and SIV-infected ART treated (SIV+/ART) macaques. B: Mesoscale Discovery S-Plex cytokine array determined the concentration of IL-6 and IL-1β per microgram of protein of DRG lysate (LDRG) from SIV− and SIV+/ART macaques. Statistical analysis was performed using a Kruskal-Wallis (KW) one-way analysis of variance and Dunn’s multiple comparison test or a U-test (UT). Data are expressed as medians ± interquartile ranges. SIV− ($n = 5$; A04-A05, A07-A09) (A), SIV+ ($n = 10$; A20-A30) (A), SIV+/ART ($n = 6$; A32-A37) (A); SIV− ($n = 5$; A04, A10-A13) (B), SIV+/ART ($n = 6$; A32-A37) (B). *P < 0.05, **P < 0.01. NS, nonsignificant.

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**Figure 2** Expression of transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1) is exclusively increased in the dorsal root ganglia (DRGs) simian immunodeficiency virus (SIV)−infected with antiretroviral therapy treated (SIV+/ART) macaques. A: Representative images from TRPV1 RNAscope DRG sections from SIV-uninfected (SIV−), SIV-infected (SIV+), and SIV+/ART macaques. Arrows indicate TRPV1 RNA-positive cells. B: The percentage of TRPV1-positive neurons in the DRG of SIV−, SIV+, and SIV+/ART macaques. C: Representative images from TRPA1 RNAscope DRG sections from SIV−, SIV+, and SIV+/ART macaques. Arrows indicate TRPA1 RNA-positive cells. D: The percentage of TRPA1-positive neurons in the DRG among groups. Statistical analysis was performed using a Kruskal-Wallis (KW) one-way analysis of variance and Dunn’s multiple comparison test. Data are expressed as medians ± interquartile ranges. SIV− ($n = 7$; A01-A03, A06-A09), SIV+ ($n = 10$; A20-A29), and SIV+/ART ($n = 6$; A32-A37) (B and D). **P < 0.01. Scale bars = 100 μm. Magnification, ×60.
TRPV1 transcripts were significantly greater in both IB4+ and TRKA+ nociceptors of simian immunodeficiency virus (SIV)–infected with antiretroviral therapy treated (SIV+/ART) macaques. A: Representative images of Opal multiplex immunohistochemistry/RNAscope targeting TRPV1 RNA (red) within IB4+ (green) or TRKA+ (pink) nociceptors in the dorsal root ganglion of SIV-uninfected (SIV−), SIV-infected (SIV+), and SIV+/ART macaques. Arrows indicate TRPV1 RNA colocalized to either a IB4+ or TRKA+ nociceptor. B and C: Percentage of either TRPV1+/IB4+ (B) or TRPV1+/TRKA+ (C) reveals colocalization of TRPV1 in IB4+ and TRKA+ nociceptors. Statistical analysis was performed using a Kruskal-Wallis (KW) one-way analysis of variance and Dunn’s multiple comparison test. Data are expressed as medians ± interquartile ranges. n = 5 SIV− macaques (A02, A05-A08); n = 6 SIV+ macaques (A20-A21, A25-A27, A29); n = 6 SIV+/ART (A32-A37) macaques (A). *P < 0.05. Scale bar = 100 μm. Magnification, ×20.

Innervation of IB4+ Primary Nociceptors into the Dorsal Horn Decreases with SIV Infection with and without ART

Although SIV infection results in retrograde dieback of DRG nerve fibers innervating the periphery,32 the dieback of the central terminals of C-fiber neurons in the spinal cord remains unexplored. The area of innervation of both IB4+ and TRKA+ nociceptors terminating in lamina 1 and 2 of the dorsal horn was visualized by immunofluorescence and compared among SIV−, SIV+, and SIV+/ART animals (Figure 4A and Supplemental Figure S6). There was a significant difference in IB4+ neuron innervation into the dorsal horn among the three groups (Kruskal-Wallis, P < 0.01, H = 11.54; Figure 4B).

IB4+ neuron innervation into the dorsal horn was significantly less in SIV+ and SIV+/ART animals compared with SIV− animals (Dunn’s multiple comparisons, P < 0.01 and P < 0.05, respectively). The study did not find any significant differences among groups in the innervation of TRKA+ neurons into the dorsal horn (Figure 4B).

Trafficking of TRPV1 to the Dorsal Horn is Limited to Innervating TRKA Neurons in SIV+/ART Animals

The protein expression of TRPV1 to the central terminals of TRKA+ nociceptors innervating in lamina 1 and IB4+ nociceptors innervating in lamina 2 of the dorsal horn was compared among the SIV−, SIV+, and SIV+/ART animals (Figure 4C and Supplemental Figure S6). There was a significant difference in the colocalization of TRPV1 to TRKA+ neurons among the three groups (Kruskal-Wallis, P < 0.05, H = 7.98; Figure 4C). The colocalization of TRPV1 was significantly greater to the innervating TRKA+ neurons in the SIV+/ART animals compared with the SIV− animals (Dunn multiple comparisons, P < 0.05;
Figure 4C). There were no significant differences in the colocalization of TRPV1 to IB4+ nociceptors in the dorsal horn among groups (Figure 4C). This finding indicates that in SIV+/ART animals there is significantly increased trafficking of TRPV1 to the central terminals of TRKA+ neurons located in lamina 1 and not to IB4+ lamina 2 neurons in the dorsal horn despite transcriptional up-regulation in both nociceptive subtypes in the DRG.

CD68+ Myeloid Neuroinflammation is Persistent in the Dorsal Horn of SIV+/ART Animals

Neuroinflammation and activation of myeloid cells can drive central sensitization, so the pan-myeloid marker CD68 that detects both macrophages and microglia was used to compare neuroinflammation in the whole LSC and the dorsal horn among the three groups (Figure 5A). The percent positive area of CD68+ cells in the whole LSC was significantly different among SIV−, SIV+, and SIV+/ART animals (Kruskal Wallis, $P < 0.05$, $H = 8.805$; Figure 5B). In the whole LSC, a significant increase in the CD68+ area was evident between SIV+ animals and SIV− controls (Dunn’s multiple comparison, $P < 0.05$; Figure 5B) and SIV+/ART animals and SIV− controls (Dunn’s multiple comparisons, $P < 0.05$). The percent positive area of CD68+ cells in the dorsal horn was significantly different among SIV−, SIV+, and SIV+/ART animals (Kruskal Wallis, $P < 0.001$, $H = 15.78$; Figure 5C). In the dorsal horn, there was significantly greater CD68+ area between SIV+ and SIV− animals (Dunn’s multiple comparisons, $P < 0.001$; Figure 5C) and between SIV+/ART and SIV− animals (Dunn’s multiple comparisons, $P < 0.05$; Figure 5C).

There is a large variation in the degree of CD68+ area among SIV-infected ART-naive animals because of different degrees of pathology and inflammation. Three of four of these animals had moderate to severe LSC myelitis and white matter degeneration (A25, A29, A30) at necropsy.
(Supplemental Table S1). Animals A25 and A29 also have a high amount of CD68$$^{+}$$ cells in the dorsal horn. The sustained increase in CD68$$^{+}$$ cells in the dorsal horn in SIV$$^{+}$$/ART animals is indicative of continuous spinal neuroinflammation despite plasma viral suppression as observed in the DRG.

**Discussion**

This study provided evidence that SIV-associated immunologic perturbations in both the peripheral nervous system (PNS) and CNS persist despite ART. Neuroinflammation from SIV infection is associated with the characteristics of neuronal sensitization, such as an up-regulation of nociceptive TRP ion channels in DRG neuron cell bodies and central terminals. To our knowledge, we are the first group to investigate changes in TRP channel expression in the PNS and CNS in SIV infection and ART. Furthermore, within the dorsal horn, neuronal and immunologic changes, such as decreased IB4$$^{+}$$ nociceptor innervation and CD68$$^{+}$$ cell inflammation, suggest loss of sensation and/or central sensitization consistent with HIV-DSP.12,13

During HIV and SIV infection, persistent monocyte/macrophage neuroinflammation contributes to neurotoxicity and functional deficits in synaptic integrity and plasticity within the CNS.14–15 Although similar neuroinflammation is observed in the DRG in people with HIV,6 there are limited data on nociceptor function and signaling in HIV-DSP. Previous studies indicate an increase in recently infiltrating MAC387$$^{+}$$ monocytes to the DRG with SIV infection, which was reduced with ART.13 However, the number of CD68$$^{+}$$ macrophages were elevated in the DRGs of SIV$$^{+}$$/ART animals relative to uninfected and SIV-infected animals. In vitro studies mimicking monocyte trafficking to the CNS during SIV infection have indicated that macrophage accumulation is driven by prevention of macrophage recirculation and protection from apoptosis.16 Although not examined here, there is published evidence that after nerve injury resident DRG macrophages proliferate locally.17 These mechanisms may play a role in the persistence of macrophages in the DRG during SIV/HIV infection and ART. Accumulation of DRG macrophages is a consistent hallmark of neuropathic pain, found in nerve injury, paclitaxel-induced, and diabetic peripheral neuropathy models.18–20 An increase in DRG macrophages after
insult to peripheral nerves was responsible for the initiation and maintenance of neuropathic pain.\textsuperscript{40–42} The release of cytokines and nuclear proteins (IL-1β, TNF-α, and high mobility group box 1 protein) implies that neuron-to-macrophage crosstalk sensitizes DRG neurons.\textsuperscript{40,41,43–45} Future studies are needed to further characterize the inflammatory output and phenotype of CD68\(^+\) cells in the DRG during SIV infection and treatment with ART. Overall, there are a limited number of SIV RNA+ cells in the DRGs with SIV infection, which was not significantly decreased with ART despite significant reduction of plasma viral load.\textsuperscript{19}

Long-term cytokine exposure derived from macrophage accumulation after nerve injury can contribute to the sensitization of DRG nociceptors by driving the expression of nociceptive ion channels.\textsuperscript{21,38,40,42} In this study, IL-6 but not IL-1β was significantly elevated in the plasma of SIV+/ART macaques from preinfection to necropsy. However, in the DRG, a higher concentration of IL-1β in SIV+/ART macaques was seen compared with uninfected animals, with no significant changes in the DRG concentration of IL-6 between groups. Because cytokines and chemokines in the peripheral blood and tissue can drive the neuroinflammatory axis, it is important to examine both compartments in HIV-DSP pathophysiology. Stimulation of primary sensory neurons with proinflammatory cytokines (IL-1β and IL-6) increased TRPV1/TRPA1 mRNA and protein levels, which was associated with increased hypersensitivity in both in vivo behavioral and in vitro functional activity.\textsuperscript{23,24,46} In this study, both TRPV1 and TRPA1 gene expression is elevated in the DRG of SIV+/ART animals compared with both uninfected and SIV+ animals. Several mechanisms may regulate TRPV1/TRPA1 expression in SIV+/ART animals, such as persistent macrophage accumulation, refractory expression from peripheral neuron regeneration, or potential sensitization effects of ART. This study was unable to delineate the potential effects of ART itself on neuronal sensitization. The clinically relevant ART regimen (tenofovir disoproxil fumarate, emtricitabine, raltegravir) did not contain dideoxynucleotide analogue reverse transcriptase or protease inhibitors, which have been associated with neuropathy and neurodegeneration.\textsuperscript{47,48}

Instead, DRG neuron atrophy was significantly decreased in SIV+/ART animals in the same cohort with SIV+ animals\textsuperscript{19} suggesting that ART intervention influences DRG neuron survival. Following insult, as in the case of untreated chronic SIV infection, DRG neurons elicit a damage response transcriptional profile diminishing their physiologic sensory functions.\textsuperscript{39} Thus, with ART intervention, nociceptors are potentially more responsive to their physiologic stimuli (eg, IL-1β). Future studies should determine the role of activated macrophages, the potential contribution of ART, and the effect of proinflammatory cytokine exposure on TRPV1/TRPA1 transcription in primary nociceptive neuron models. Although an increase in relative RNA expression of TRPV1/TRPA1 across the animal conditions was observed, single-cell transcriptomic studies have identified a higher percentage of TRPV1-RNA+ DRG neurons in SIV-uninfected macaques.\textsuperscript{50} Because this study was designed as a cross-sectional retrospective approach, it was limited in its ability to perform these more highly sensitive techniques. Despite this, transcriptomic and translational-level data follow an increasing trend of TRPV1 expression, particularly in SIV+/ART macaques. Future single-cell RNA-sequencing studies of DRG neurons from uninfected, SIV-infected, and SIV+/ART macaques should determine perturbations in the functional landscape based on expression of nociceptive ion channels that can contribute to maintenance of HIV-DSP.

The primary DRG neuronal subset of interest in neuropathic pain conditions are the C-fiber nociceptors.\textsuperscript{51–53} Atrophy and a decrease in the number of both TRKA+ and IB4+ nociceptors occurs with SIV infection and an improvement in IB4+ neuron density is seen with ART.\textsuperscript{19} This study shows an increase in TRPV1 transcription in both the IB4+ and TRKA+ nociceptors in SIV+/ART animals compared with uninfected controls. TRPV1/TRPA1 expression by nociceptive subtype can be defined not only by physiologic expression\textsuperscript{44} but also transcriptional switches after chronic inflammation and nerve injury from mainly TRKA+ peptidergic nociceptors to IB4+ nonpeptidergic nociceptors.\textsuperscript{55,56} Because there is improved IB4+ neuron survival with ART,\textsuperscript{19} the contributions of not only previously injured but uninjured neurons need to be considered as well. Following a partial spinal nerve ligation, injured neurons decrease TRPV1 expression, whereas uninjured DRG neurons increase TRPV1 expression.\textsuperscript{57,58} Spared uninjured C-fiber nociceptors after injury have an increased rate of spontaneous activity that is associated with severity of pain behaviors.\textsuperscript{59–61} Herein, chronic DRG inflammation led to increased TRPV1/TRPA1 transcription in both C-fiber nociceptive subsets in SIV+/ART animals. Future studies should examine whether TRPV1 expression among nociceptive subtypes is attributed to nociceptor phenotypic switching and the extent of contributions from regenerating versus uninjured nociceptors during chronic inflammation. Finally, this study cannot directly link an increase in TRPV1/TRPA1 transcription to neuronal firing to determine the effects of downstream signaling cascades after TRPV1/TRPA1 activation. The downstream signaling of TRPV1 activation is not limited to Ca\(^{2+}\)-dependent peripheral sensitization and thus can act through modulation of TRPA1 or the release of neuropeptides contributing to inflammatory-mediated hyperalgesia.\textsuperscript{62,63} Furthermore, in a model of diabetic peripheral neuropathy, Ca\(^{2+}\) influx through the TRPV1 channel increased oxidative stress, mitochondrial reactive oxygen species production, and markers of apoptosis in the DRG.\textsuperscript{64} These stressors can culminate in DRG neuron cytotoxicity, which can be reversed by one of many TRPV1-targeted treatments.\textsuperscript{62,64} Therapeutic targeting of TRPV1 function is associated with neuropathic pain relief in which desensitization
through agonism and functional blockade through antagonism are well characterized.\textsuperscript{62,65} Future studies should determine the downstream signaling implications of increased TRPV1 transcription and activation potential in the context of maintaining peripheral sensitization and DRG neuron cytotoxicity.

Acute pain sensitization is driven by changes in peripheral nociceptive signaling in the DRG, whereas CNS sensitization is necessary for development of chronic neuropathy.\textsuperscript{66,67} Trafficking of TRP ion channels at the protein level to the dorsal horn plays a key role in the development of central sensitization through SNARE protein recycling\textsuperscript{27} and synaptic vesicle release.\textsuperscript{68} In this study, TRPV1 was localized to the central terminals of TRKA+, but not IB4+ neurons, in SIV+/ART animals. Therefore, increased trafficking of TRPV1/TRPA1 to the dorsal horn resulting from SIV-associated DRG inflammation could increase neuropeptide release and action potential production, contributing to central sensitization in HIV-DSP.\textsuperscript{21,28} In addition, reinnervation of nociceptive subtypes into the dorsal horn may play a key role in dorsal horn localization.

Retrograde degeneration of distal sensory afferents is well described in both HIV-DSP\textsuperscript{66,69} and SIV infection.\textsuperscript{13} Despite this and evidence of DRG cell body damage, the effects SIV infection on DRG neuron innervation in the dorsal horn have not been investigated. Herein, a decrease in IB4+ neuron innervation into the dorsal horn in SIV+ and SIV+/ART animals relative to uninfected animals was observed. There were no differences in TRKA+ innervation among groups. These data indicate deficits in IB4+ terminals in the dorsal horn of the spinal cord, whereas TRKA+ nociceptors have deficits in the DRG. Previous reports indicate cell body regeneration may not always mirror the regenerative capacity of distal axons.\textsuperscript{70} After nerve injury, distal branches of damaged TRKA+ nociceptors innervating the skin increase terminal branching—inducing hyperinnervation.\textsuperscript{71,72} We hypothesize that the differences in nociceptor density in the DRG and terminal innervation into the dorsal horn could be due to antiregenerative environment of the PNS/CNS border and axonal branching of TRKA+ nociceptors amid DRG damage and terminal atrophy.\textsuperscript{68,73} The loss of DRG central terminals innervating the dorsal horn\textsuperscript{74} results in loss of sensation and formation of pathologic synaptic connections, whereas hyperinnervation of TRKA+ nociceptors is associated with neuronal hyperexcitability.\textsuperscript{71–73,75} Degeneration and hyperinnervation of distal DRG terminals are well-studied in the context of neuropathic pain after PNS injury, with little focus on proximal innervation. Additional studies should identify molecular mechanisms perturbing dorsal horn innervation from DRG-derived terminals during SIV infection/ART and the role denervation of the dorsal horn plays in manifestation of HIV-DSP and other neuropathic pain models.

Neuroinflammation and specifically microglial activation in the dorsal horn can lead to mechanisms of central pain sensitization.\textsuperscript{28} SIV infection with and without ART led to an increase in the number of CD68\textsuperscript{+} cells in the DRG and the spinal cord. There was also a higher number of CD68\textsuperscript{+} cells specifically in the dorsal horns of SIV+ and SIV+/ART macaques. Microglial activation is a hallmark of a CNS response to peripheral nerve injury and/or SIV infection.\textsuperscript{30,76} Spinal microglia activation alters the activity of the K\textsuperscript{+}−Cl\textsuperscript{−} cotransporter, KCC2, on second-order dorsal horn neurons, leading to a loss of tonic inhibitory control in the dorsal horn.\textsuperscript{76–79} CD68\textsuperscript{+} cells in the dorsal horn may also play a role in the response to tissue damage, such as central terminal atrophy. In addition, microglial accumulation in the superficial dorsal horn engulf\textsuperscript{+} particularly inhibitory synaptic receptors in innervating C-fiber nociceptors after peripheral nerve injury perpetuating central sensitization.\textsuperscript{80} Future studies should further define the CD68\textsuperscript{+} population in the dorsal horn in terms of cellular subtypes, activation states, and functional output and how this relates to disinhibition in the dorsal horn.

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Author Contributions

R.W., J.A.R., and T.H.B. conceptualized and designed the study; R.W. collected data; A.D.M. assessed animal pathology; R.W., J.A.R., R.M.P., and T.H.B. analyzed data; R.W., J.A.R., and T.H.B. edited the manuscript; and R.W. and T.H.B. wrote the manuscript.

Disclosure Statement

T.H.B. holds equity and is a member of the scientific advisory board of Excision BioTherapeutics Inc. unrelated to this project. R.W. has received travel awards to present this work at International Society for Neuromicrobiology conference in June 2021 and Society on Neuroimmune Pharmacology conference in June 2022. All other authors declare no conflict of interests.

Supplemental Data

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