University of New England
DUNE: DigitalUNE

Biomedical Sciences Faculty Publications | Biomedical Sciences Faculty Works

8-18-2009

Long Lasting Recruitment Of Immune cells And Altered Epiperineurial Thickness In Focal Nerve Inflammation Induced By Complete Freund's Adjuvant

Geoffrey M. Bove
University of New England, gbove@une.edu

Follow this and additional works at: http://dune.une.edu/biomed_facpubs

Part of the Medical Sciences Commons

Recommended Citation
Bove, Geoffrey M., "Long Lasting Recruitment Of Immune cells And Altered Epiperineurial Thickness In Focal Nerve Inflammation Induced By Complete Freund's Adjuvant" (2009). *Biomedical Sciences Faculty Publications*. 10.
http://dune.une.edu/biomed_facpubs/10

This Article is brought to you for free and open access by the Biomedical Sciences Faculty Works at DUNE: DigitalUNE. It has been accepted for inclusion in Biomedical Sciences Faculty Publications by an authorized administrator of DUNE: DigitalUNE. For more information, please contact bkenyon@une.edu.

NIH Public Access

Author Manuscript

J Neuroimmunol. Author manuscript; available in PMC 2013 July 13.

Published in final edited form as:
J Neuroimmunol. 2009 August 18; 213(0): 26–30. doi:10.1016/j.jneuroim.2009.06.005.

Long lasting recruitment of immune cells and altered epi-perineurial thickness in focal nerve inflammation induced by complete Freund's adjuvant

Geoffrey M. Bove[a,*], **Wendy Weissner**[b], and **Mary F. Barbe**[c,d]

[a]Dept. of Research Administration, University of Southern Maine, 178 Science Building, P.O. Box 9300, Portland, ME 04104-9300, United States

[b]Natural Standard Research Collaboration, One Davis Square, Somerville, MA 02144, United States

[c]Department of Physical Therapy, Temple University, 3307 North Broad St., Philadelphia, PA 19140, United States

[d]Department of Anatomy and Cell Biology, Temple University Medical School, 3400 North Broad St., Philadelphia, PA 19140, United States

Abstract

Immune-mediated nerve inflammation is involved in many painful states in humans, and causes axonal and behavioral changes in rats. While models of nerve inflammation have been characterized using electrophysiological and behavioral methods, the presence of immune cells has not been fully assessed. We inflamed rat sciatic nerves using complete Freund's adjuvant and quantified the presence of ED-1 macrophages and TCR-αβ T-cells for up to 12 weeks. We report that these immune cells are prominent extraneurally up to 12 weeks following the induction of inflammation. This observation does not easily correlate with inflammation-induced axonal mechanical sensitivity, which peaks within 1 week and is resolved after 8 weeks.

Keywords

Neuritis; Peripheral nerve; Macrophages; T-cells; Radiating pain

1. Introduction

Immune-mediated nerve inflammation has been proposed as the mechanism behind symptoms of perineuritis, diabetic neuropathy, endoneurial vasculitis, radiculopathies, and paraneoplastic peripheral neuropathy (Asbury et al., 1972; Bourque et al., 1985; Cain et al., 2001; Chacur et al., 2001; Said, 1995; Said et al., 1997; Smitt and Posner, 1995; Sorenson et al., 1997). The presentations of pain from all these causes are similar, and the common denominator is inflammation of the involved nerve.

We model focal nerve inflammation by the application of complete Freund's adjuvant (CFA) to the sciatic nerves of rats. Within days, this induces a robust focal inflammation, or neuritis. The inflammation is characterized by a mass around the nerve that contains a large number of macrophages (Bove et al., 2003). We have also shown that CFA-induced neuritis

© 2009 Elsevier B.V. All rights reserved

*Corresponding author. Tel.: +1 207 228 8075 (office); fax: +1 207 228 8057. geoffreybove@gmail.com (G.M. Bove)..

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

causes the axons of many mechanically sensitive nociceptors to develop ectopic mechanical sensitivity, at the site of inflammation. The axonal mechanical sensitivity (AMS) during CFA-neuritis peaks at 4–7 days (Bove et al., 2003; Dilley et al., 2005), and resolves by 8 weeks (Dilley and Bove, 2008b). However, the time course of immune cell presence has not been described.

ED-1 macrophages and TCR-αβ T-cells release a myriad of substance that can be pro-inflammatory, tissue damaging, and noxious (Al Shatti et al., 2005; Huse et al., 2008; Nathan, 1987), which may be responsible for the development of AMS. We hypothesized that if the presence of immune cells is required for the development of AMS, then their numbers would increase and decrease in conjunction to AMS. This study was designed to determine the time course of ED-1 positive macrophage and TCR-αβ T-cell presence in the CFA-neuritis model.

2. Materials and methods

Experiments were carried out using adult male Wistar rats weighing 250–300 g. All procedures were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center and were in accordance with the guidelines of the International Association for the Study of Pain.

Anesthesia was induced using 2.5–3% isoflurane in oxygen, and maintained with 1.7–2% isoflurane in oxygen. Neuritis was induced as previously described (Bove et al., 2003). Using aseptic technique, the skin over the mid-thigh was cut, and the muscles were dissected to reveal the sciatic nerve. The nerve was gently freed from underlying tissue for 6–7 mm and a 3×3×10 mm piece of absorbable gelatin sponge (GelFoam, Pharmacia & Upjohn, USA) saturated in the test compound [complete Freund's adjuvant (CFA, Sigma) or sterile saline (sham)] was wrapped around the nerve. The dose of Mycobacterium tuberculosis debris in the CFA experiments was ~75 μg. Controls were the contralateral, unaffected nerves. The incisions were closed in layers and the animals were allowed to recover. After surgery, rats were assigned to survival times of 2 days, and 1, 4, 8, and 12 weeks. Groups consisted of 4 rats each, and 44 rats were used in total (20 CFA-treated, 20 sham-treated, and 4 untreated).

For immunohistochemistry, rats were anesthetized with an overdose of sodium pentobarbital (200 mg/kg, i.p.) and perfused transcardially with heparinized 0.1 M phosphate buffered saline (PBS; pH 7.4). The ipsilateral and contralateral sciatic nerves and the surrounding inflammatory complexes were harvested and flash frozen in mounting medium in a well of isobutane chilled by dry ice. The nerves were cross-sectioned using a cryostat (8 μm) and then thaw-mounted on glass slides. Sections were immediately fixed in 4% paraformaldehyde for 7 min, rinsed in PBS and then in distilled water. Slides were then placed in a 1% H_2O_2/methanol solution for 30 min to block endogenous peroxidase, and rinsed in distilled water followed by PBS. Slides were blocked with 4% normal goat serum in PBS and reacted with specific antibodies against ED-1 (Serotec, USA; 1:250) or TCR-αβ (Pharmingen, USA; 1:25) overnight at 4 °C. Slides were washed in PBS, blocked for 30 min in 4% normal goat serum in PBS, and reacted with biotin-SP-conjugated goat anti-mouse IgG secondary (Jackson Labs, USA; 1:500) for 30 min. Product visualization was achieved with the ABC Elite Kit (Vector Labs, USA; USA) for 30 min, followed by DAB (Vector Labs, USA) for 12 min. Sections were rinsed in distilled water for 10 min, counterstained with H&E, dehydrated with ascending concentrations of alcohol, cleared with xylene, and coverslipped with DPX (Biochemika, Switzerland). Control slides were processed without the primary antibody.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Nerve sections were analyzed quantitatively for the presence of immunolabeled immune cells using a Nikon microscope interfaced with a quantitation system (Bioquant Osteo II, Bioquant, TN). The identity of the sections was concealed for analysis. A representative section of each of 3 sections of each nerve, separated by at least 40 μm, were analyzed at 100x. Based on preliminary observation of the sections, which had a strongly eosinophilic region, we determined that dividing the inflamed complex into inner and outer zones was most appropriate for analysis. First, a region of interest (ROI) was drawn to include the eosinophilic epi-perineurium (inner ROI; Fig. 1C). The optical characteristics of the pixels representing the immunolabeled immune cells within the ROI were determined (Al Shatti et al., 2005). For each section, the spectrum of color representing the immunolabel was determined by the operator by choosing individual pixels and then applying the label (in red) until all the visually apparent immunolabel was selected (as in Figs. 1 and 2). The number of pixels that were chosen in this manner were counted and then compared to the total number of pixels in the ROI, thus calculating the percentage of ED-1 or TCR-αβ immunolabeling within the ROI. Next, another ROI was drawn from the outer margin of the inner ROI to include up to 250 μm of the inflammatory complex beyond the epi-perineurium (outer ROI; Fig. 1D and E), and the percentage of labeled pixels similarly determined. It was noted that the epineurium and perineurium were difficult to differentiate histologically, especially following CFA application, and therefore, they were here termed "epi-perineurium." This distinctly eosinophilic epi-perineurium appeared thicker following the experimental manipulations. Therefore, the inner and outer margins of the eosinophilic zone were used to calculate the average thickness of the epi-perineurium (Fig. 1F). Two-way ANOVAs were used to analyze the pixel-percent of ED-1 and TCR-αβ immunolabeling as well as the perineurial thickness measurements, using treatment, group, and interaction as variables. Bonferroni post-hoc tests were used when significant group effects were detected. *p*-values below 0.05 are reported as significant.

3. Results

3.1. General observations

As we have previously observed (Bove et al., 2003), the application of CFA to the nerves caused a robust inflammatory reaction, indicated by swelling of the lesion area and influx of immune cells (Figs. 1 and 2). Interestingly, the application of saline in GelFoam also led to grossly similar appearances. A striking observation was that at no time in either model did immune cells appear to penetrate the perineurium to reside within the fascicle of the nerve. The cells remained in the outer parts of the eosinophilic epi-perineurium (Figs. 1 and 2). At 4W, 8W, and 12W, the inflamed matrix developed a layer of fibrotic tissue exterior to the lesion, more notably in the CFA group (Fig. 1E–G, arrows).

3.2. Immune cells

Chronologically, the patterns of ED-1 immunolabeled macrophages and TCR-αβ immunolabeled T-cell presence were very similar (Fig. 3). When inner-ROI and outer-ROI zones were combined (Fig. 3A and D), the percentages of pixels with immunolabel for ED-1 were significantly higher ($p<0.05$, AVOVA) than control at 4W, 8W, and 12W, and higher than sham at 4W and 8W. For TCR-αβ, significant increases from control were observed at 4W, 8W, and 12W, and from sham at 4W and 12W. The percentage of ED-1 and TCR-αβ immunolabeling appeared to be decreasing at 12 weeks, but was still significantly higher than controls, indicating that the post-inflammatory effects of the lesion were still being resolved. The percentage of ED-1 and TCR-αβ immunolabeling in the outer zone in general followed the pattern of both zones combined (Fig. 3B and E). When the inner ROI (epi-perineurial zone) was analyzed separately, no significant changes in immunolabeling were apparent at any time point for either cell type (Fig. 3C and F). This observation indicates

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

that cells were largely excluded from the epi-perineurium as well as from the nerve inside of the perineurium.

3.3. Epi-perineurial measurements

In both neuritis and sham groups, the epi-perineuria appeared edematous in the first weeks following surgery (Figs.1 and 2), and then later appeared consolidated and thickened, indicated by the presence of more dense fibrous tissue (Figs. 1E–H and 2C–D). Epi-perineurial thickness measurements from unoperated animals were 36±2.4 μm (Fig. 4). In both the neuritis group and the sham group there were significant thickness increases at all postoperative time points ($p<0.01$; ANOVA), with the exception of the 2D and 12W sham groups. The epi-perineurial thicknesses were significantly increased ($p<0.05$) in the neuritis group compared to sham treatment at 1W, 8W, and 12W. The thickness of the perineuria in the sham-treated nerves was not significantly different at 12W compared to control nerves.

4. Discussion

The main objective of this project was to determine whether immune cell presence was related to the etiology of inflammation-induced axonal mechanical sensitivity (AMS). Because the maximum effect of inflammation on axons in terms of AMS is within one week (Dilley and Bove, 2008b), the concept that this phenomenon is directly related to the compounds released by macrophages and T-cells is not supported. However, it remains possible that there are other subtypes of macrophages or T-cells that are recruited at different times during inflammation. While such subtypes could produce different factors, and thus have different effects over time, such dynamics have not been reported.

Macrophages and T-cells secrete a myriad of chemicals that are known to injure cell membranes and cause pain symptoms (Nathan, 1987). Although the perineurium has been reported to be resistant to inflammatory mediators (Olsson, 1990; Rydevik et al., 1985; Todd et al., 1997), others and we have shown that TNF-α, which is released by macrophages, induces ongoing activity in nociceptors when applied to intact nerves (Leem and Bove, 2002; Sorkin et al., 1997), supporting that this cytokine penetrates the perineurium of normal nerves. Our data clearly demonstrate altered epi-perineuria at all postoperative times, but our analysis did not include perineurial functional measurements. It is possible that the perineurium retains its barrier functions during inflammation, and this is supported by our findings that the subsets of immune cells examined never penetrated the perineurium.

Using the same neuritis model, we have also reported that AMS and ongoing activity (non-evoked discharge) may occur separately or together in regionally inflamed nociceptor axons (Bove et al., 2003). Therefore, it remains possible that the mechanism of action of TNF-α or other inflammatory components on ongoing activity is independent of AMS. The current findings further support that the mechanisms of induction of AMS and ongoing activity are different, and that AMS may not be directly induced by inflammatory mediators.

One clue to the etiology of AMS is our recent finding showing that slowed axoplasmic transport, in the absence of inflammation, leads to AMS (Dilley and Bove, 2008a). Axoplasmic transport is slowed during inflammation (Amano et al., 2001; Armstrong et al., 2004), but by unknown factors. In-vitro, CFA nerve inflammation caused a histamine-dependent reduction in axoplasmic flow. Additionally, inflammation increases intraneural pressure (Gelberman et al., 1981), especially when nerves pass through constrictions. Pressure alone has been shown to slow axoplasmic transport, and nerves under chronic pressure are mechanically sensitive (Greening et al., 2005), consistent with our findings of axonal mechanical sensitivity. It is possible that a factor not directly mediated by ED-1 or TCR-αβ immune cells, such as mechanical pressure, reduces axoplasmic flow and causes

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

AMS. Intrafascicular pressure measurements during nerve inflammation, which are currently lacking, would provide supportive evidence for this possibility.

In summary, focal immune-mediated nerve inflammation caused a long lasting influx of ED-1 macrophages and TCR-αβ T-cells. Sterile saline treatment also caused an influx of both subsets of immune cells that paralleled CFA-induced inflammation up to 1 week, though the changes never reached statistical significance. Importantly, these immune cells never penetrated the perineurium; that is, inflammation did not lead to their penetration into the nerve. While it is evident from other studies that the axons within a nerve are affected by inflammatory responses surrounding the nerve, this effect must either due to diffusible inflammatory factors, due to factors not directly related to the inflammation, or both. Because the maximum effect of inflammation on axons in terms of AMS is within one week (Dilley and Bove, 2008b), the concept that this phenomenon is directly related to the compounds released by ED-1 macrophages and TCR-αβ T-cells is not supported. However, it remains possible that undetermined differential release patterns of cytokines or other mediators from these or other immune cells could be responsible for the physiological changes observed during neuritis. It also remains possible that inflammation affects axons indirectly, such as through the effects of decreased axoplasmic flow or increased intrafascicular pressure.

Acknowledgments

Financial support was provided by National Institutes of Health Grant 5R01AR048925, though the National Institute of Arthritis and Musculoskeletal and Skin Diseases and the National Center for Complementary and Alternative Medicine.

This work was supported by a grant to GMB by the Samueli Institute. The views, opinions and/or findings contained in this report are those of the author and should not be construed as the opinion or policy of the Samueli Institute. This work was also supported by the US Army Medical Research and Materiel Command under Award No. W81XWH-06-1-0279. The views, opinions and/or findings contained in this report are those of the author and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation. The preceding wording is used to satisfy contractual obligations with the Samueli Institute and the US military.

References

Al Shatti T, Barr AE, Safadi FF, Amin M, Barbe MF. Increase in inflammatory cytokines in median nerves in a rat model of repetitive motion injury. J. Neuroimmunol. 2005; 167:13–22. [PubMed: 16026858]

Amano R, Hiruma H, Nishida S, Kawakami T, Shimizu K. Inhibitory effect of histamine on axonal transport in cultured mouse dorsal root ganglion neurons. Neurosci. Res. 2001; 41:201–206. [PubMed: 11591447]

Armstrong BD, Hu Z, Abad C, Yamamoto M, Rodriguez WI, Cheng J, Lee M, Chhith S, Gomariz RP, Waschek JA. Induction of neuropeptide gene expression and blockade of retrograde transport in facial motor neurons following local peripheral nerve inflammation in severe combined immunodeficiency and BALB/C mice. Neuroscience. 2004; 129:93–99. [PubMed: 15489032]

Asbury AK, Picard EH, Baringer JR. Sensory perineuritis. Arch. Neurol. 1972; 26:302–312. [PubMed: 5015590]

Bourque CN, Anderson BA, Martin DC, Sima AA. Sensorimotor perineuritis—an autoimmune disease? Can. J. Neurol. Sci. 1985; 12:129–133. [PubMed: 3893664]

Bove GM, Ransil BJ, Lin H-C, Leem JG. Inflammation induces ectopic mechanical sensitivity in axons of nociceptors innervating deep tissues. J. Neurophysiol. 2003; 90:1949–1955. [PubMed: 12724363]

Cain DM, Wacnik PW, Turner M, Wendelschafer-Crabb G, Kennedy WR, Wilcox GL, Simone DA. Functional interactions between tumor and peripheral nerve: changes in excitability and

morphology of primary afferent fibers in a murine model of cancer pain. J. Neurosci. 2001; 21:9367–9376. [PubMed: 11717370]

Chacur M, Milligan ED, Gazda LS, Armstrong C, Wang HC, Tracey KJ, Maier SF, Watkins LR. A new model of sciatic inflammatory neuritis (SIN): induction of unilateral and bilateral mechanical allodynia following acute unilateral perisciatic immune activation in rats. Pain. 2001; 94:231–244. [PubMed: 11731060]

Dilley A, Bove GM. Disruption of axoplasmic transport induces mechanical sensitivity in intact rat C-fibre nociceptor axons. J. Physiol. 2008a; 586:593–604. [PubMed: 18006580]

Dilley A, Bove GM. Resolution of inflammation induced axonal mechanical sensitivity and conduction slowing in C-fiber nociceptors. J. Pain. 2008b; 9:185–192. [PubMed: 18309534]

Dilley A, Lynn B, Pang SJ. Pressure and stretch mechanosensitivity of peripheral nerve fibres following local inflammation of the nerve trunk. Pain. 2005; 117:462–472. [PubMed: 16154692]

Gelberman RH, Hergenroeder PT, Hargens AR, Lundborg GN, Akeson WH. The carpal tunnel syndrome. A study of carpal canal pressures. J. Bone Jt. Surg., Am. 1981; 63:380–383.

Greening J, Dilley A, Lynn B. In vivo study of nerve movement and mechanosensitivity of the median nerve in whiplash and non-specific arm pain patients. Pain. 2005; 115:248–253. [PubMed: 15911151]

Huse M, Quann EJ, Davis MM. Shouts, whispers and the kiss of death: directional secretion in T cells. Nat. Immunol. 2008; 9:1105–1111. [PubMed: 18800163]

Leem JG, Bove GM. Mid-axonal tumor necrosis factor-alpha induces ectopic activity in a subset of slowly conducting cutaneous and deep afferent neurons. J. Pain. 2002; 3:45–49. [PubMed: 14622853]

Nathan CF. Secretory products of macrophages. J. Clin. Invest. 1987; 79:319–326. [PubMed: 3543052]

Olsson Y. Microenvironment of the peripheral nervous system under normal and pathological conditions. Crit. Rev. Neurobiol. 1990; 5:265–309. [PubMed: 2168810]

Rydevik B, Brown MD, Ehira T, Nordborg C. Effects of collagenase on nerve tissue. An experimental study on acute and long-term effects in rabbits. Spine. 1985; 10:562–566. [PubMed: 3001946]

Said G. Vasculitic neuropathy. Bailliere's Clin. Neurol. 1995; 4:489–503. [PubMed: 8599722]

Said G, Elgrably F, Lacroix C, Plante V, Talamon C, Adams D, Tager M, Slama G. Painful proximal diabetic neuropathy: inflammatory nerve lesions and spontaneous favorable outcome. Ann. Neurol. 1997; 41:762–770. [PubMed: 9189037]

Smitt PS, Posner JB. Paraneoplastic peripheral neuropathy. Bailliere's Clin. Neurol. 1995; 4:443–468. [PubMed: 8599720]

Sorenson EJ, Sima AA, Blaivas M, Sawchuk K, Wald JJ. Clinical features of perineuritis. Muscle Nerve. 1997; 20:1153–1157. [PubMed: 9270672]

Sorkin LS, Xiao W-H, Wagner R, Myers RR. Tumor necrosis factor-α induces ectopic activity in nociceptive primary afferent fibers. Neuroscience. 1997:255–262. [PubMed: 9300418]

Todd BA, Sedgwick EM, Abbott NJ. Effects of the bile salt sodium deoxycholate, protamine, and inflammatory mediators on the potassium permeability of the frog nerve perineurium. Brain Res. 1997; 776:214–221. [PubMed: 9439815]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

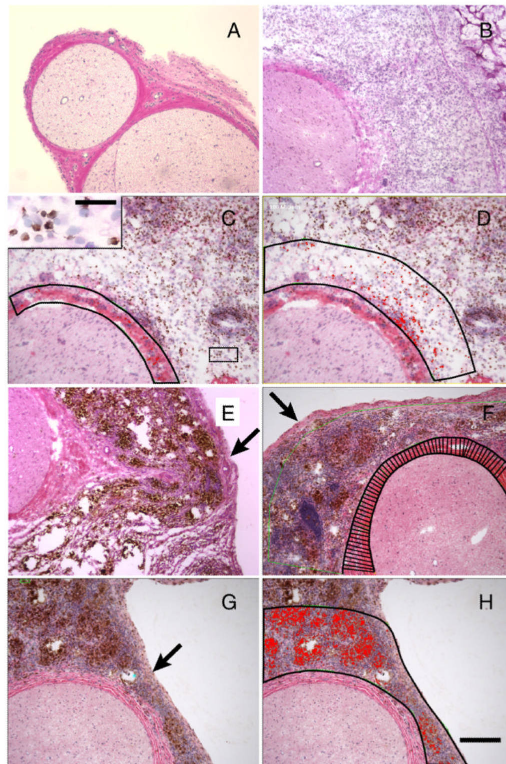

Fig. 1.

Representative images (cross-sections) for unreacted, ED-1 immunolabel, and quantification methods. A. Normal nerve. B. One week (1W) neuritis following surgery. Note swelling compared to A, restricted to outer parts of the section. This is also a control slide for immunolabeling (= no primary antibody). C and D (same section as C). 1W neuritis is characterized by substantial swelling. The definitions of the inner and outer regions of interest (ROI) are shown, as well as examples of immunolabel thresholding (red dots; inner ROI in C, outer ROI in D). Inset in C is higher power image of box showing ED-1 immunolabeled cells (scale bar=50 µm). (E). 4W neuritis. Arrow indicates fibrosis of the outermost part of the complex, also seen at 8W (F) and 12W (G). When present, the fibrosis formed a convenient plane for sample removal. F. 8W neuritis, showing consolidation of immunolabel. Also shown is the method used to measure the thickness of the epi-perineurium. G. 12W neuritis. H. Same section as G, showing example of immunolabel thresholding. All images counterstained with H&E. Scale bar=200 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

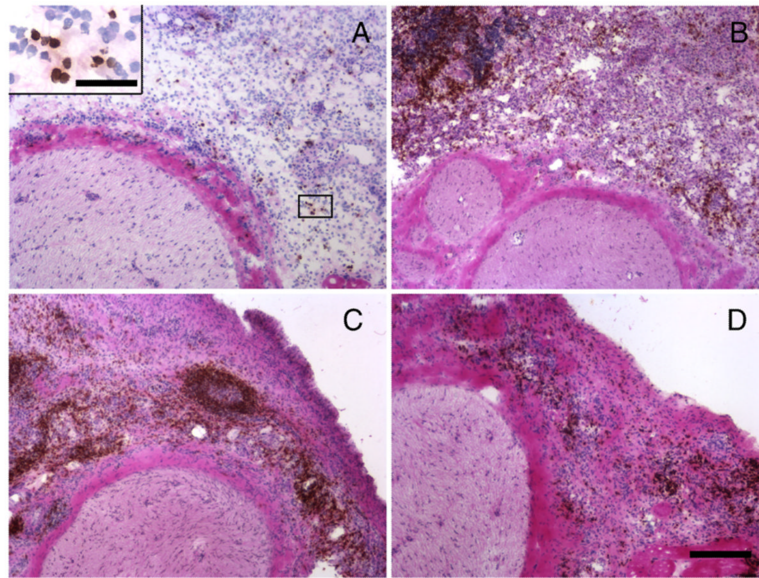

Fig. 2.
Representative images (cross-sections) for TCR-αβ immunolabel, at 1W (A), 4W (B), 8W (C), and 12W (D). A is from an adjacent section of Fig. 1C. Inset in A is higher power image of box showing TCR-αβ immunolabeled cells (scale bar=50 μm). All images counterstained with H&E. Scale bar=200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

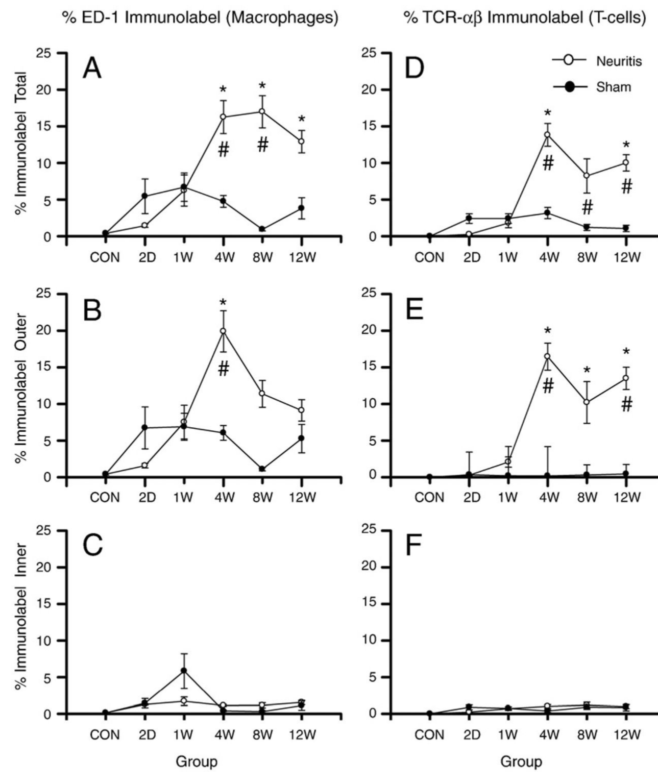

Fig. 3.

Percent area of ED-1 immunolabel for macrophages and TCR-αβ immunolabel for T-cells during neuritis, over time. A–C: Percent area of ED-1 immunolabel (macrophages) in the combined (A), outer (B), and inner (C) zones. D–F: Percent area of TCR-αβ immunolabel (T-cells) in the combined (C), outer (D), and inner (E) zones. * = significant by ANOVA from saline treatment (sham). # = significant by ANOVA from control.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

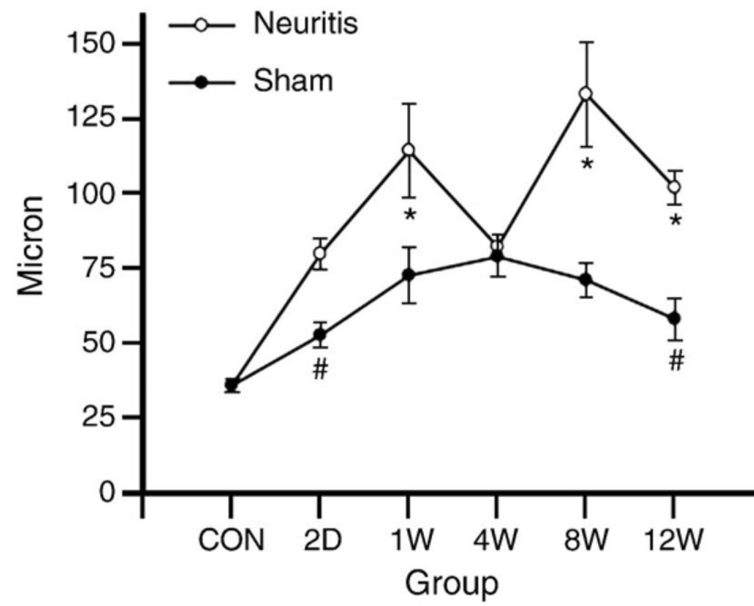

Fig. 4.
Epi-perineurial thickness measurements. Both neuritis and sham led to significant increases in thickness compared to control at all time points, except for 2D and 12W sham (#). Neuritis caused greater increases than sham at 2W, 8W, and 12W (*).