B cell Activity and anti-MBP Autoantibody Response to Peripheral Nerve Injury is Sexually Dimorphic

Hee Jong Lee  
UCSD: University of California San Diego

Albert Remacle  
Sanford-Burnham Medical Research Institute: Sanford Burnham Prebys Medical Discovery Institute

Swathi Hullugundi  
UCSD: University of California San Diego

Jennifer Dolkas  
UCSD: University of California San Diego

Jake Leung  
UCSD: University of California San Diego

Andrei Chernov  
UCSD: University of California San Diego

Tony Yaksh  
UCSD: University of California San Diego

Alex Strongin  
UCSD: University of California San Diego

Veronica Shubayev  
University of California San Diego  
mailto:vshubayev@ucsd.edu  
https://orcid.org/0000-0003-2598-263X

Research

**Keywords:** Autoantibodies, disorders, pathogenesis , CD20 immunoblotting

**DOI:** https://doi.org/10.21203/rs.3.rs-117432/v1

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

**Background:** Autoantibodies, a hallmark of autoimmune disorders, are thought to contribute to the pathogenesis of chronic pain. Mechanical hypersensitivity is a severe complication of peripheral nerve injury mediated through sex-specific activity of immune and glial cells. Whether B cell contribution to nerve injury and pain is sexually dimorphic remains unknown.

**Methods:** CD20 immunoblotting and immunostaining was conducted in female and male sciatic nerve after chronic constriction injury (CCI). B cell/CD20 targeting by bolus intravenous (IV) Rituximab or control immunoglobulin (IVIG) therapy or vehicle was administered in female and male rats post-CCI, followed by von Frey mechanical sensitivity testing. The cryptic myelin basic protein (MBP) epitopes were identified by immunostaining (EMD-AB5864 Millipore antibody) in female and male CCI nerves. ELISA was used to detect serum autoantibodies against the immunodominant MBP(84-104) epitope was conducted in female and male rats post-CCI.

**Results:** Infiltration of CD20-positive cells, presumably B cell, was observed in female and male nerve at one week post-CCI. A single bolus IV anti-CD20/Rituximab therapy at one week post-CCI reduced the degree of mechanical hypersensitivity in female, but not in male, rats. IVIG therapy and vehicle treatment had no effect in either sex. Sustained release of the cryptic MBP epitopes from myelin was observed in nerves post-CCI in both sexes. Remarkably, serum anti-MBP(84-104) autoantibody was detected in female, but not in male, rats post-CCI.

**Conclusions:** B cell activity and autoantibody production after peripheral nerve injury are sexually dimorphic. In females, mechanical hypersensitivity arising due to autoimmune remodeling of myelin on sensory neuraxis is amenable to immunotherapeutic intervention.

Introduction

Chronic pain [1, 2] and autoimmune conditions [3, 4] are major public health concerns, both prevalent in women. Certain chronic pain states are thought to represent an autoantibody-mediated autoimmune response targeted to afferent neurons [5–9]. Injury to the peripheral nervous system (PNS) produces mechanical hypersensitivity states, including a paradoxical pain from light tactile stimulation (mechanical allodynia), which may sustain for years after injury incitement. Neuroinflammation [10–12], including adaptive, T cell-mediated immune signaling [13–20], has long been implicated in the development of neuropathic pain states. Recent evidence in rodents indicated that T cells mediate mechanical hypersensitivity selectively in female, but not in male, after PNS injury [21]. T cell activity requires presentation of discrete epitopes by antigen presenting cells, including B cells. The specific epitopes involved in sex-specific regulation of mechanical hypersensitivity after PNS trauma and the role of B cells in sexual dimorphism of neuropathic pain remain obscure.

In the PNS, mechanosensory neurons are insulated by myelin sheath, in which myelin basic protein (MBP) is a putative autoantigen. Proteolytic release of cryptic immunodominant MBP epitopes
contributes to autoimmune demyelinating pathologies, including multiple sclerosis and Guillain-Barre syndrome [22, 23]. We have demonstrated that mechanical hypersensitivity associated with demyelination after a focal PNS trauma [24–27] is due in part to the targeted activity of the cryptic immunodominant MBP epitopes [14, 28–30]. We have shown that the peptides comprising the conserved α-helix 87-VVHFF-91 of MBP motif, including MBP(68–86) and MBP(84–104) peptides, administered by a bolus adjuvant-free intraneural (IN) injection into an intact sciatic nerve, trigger a sustained (weeks), T cell-dependent mechanical hypersensitivity in female rats [14, 28, 30, 31]. Blocking MBP epitope release using metalloproteinase inhibitors [14, 28, 29] or MBP epitope activity by active immunization with the altered/mutant MBP ligand [32] attenuates mechanical allodynia associated with sciatic nerve chronic constriction injury (CCI).

Rituximab (RTX) is a human-mouse chimeric monoclonal antibody against cell surface CD20 glycoprotein licensed for medical use [33]. Intravenous (IV) RTX improves management of autoimmune diseases, including rheumatoid arthritis and systemic lupus erythematosus, by antibody- and complement-dependent B cell cytotoxicity [34]. In addition, IV RTX shows efficacy in mitigating pain in patients with complex regional pain syndrome (CRPS) [35] and anti-myelin associated glycoprotein (MAG) IgM demyelinating neuropathy [36]. While a human-mouse chimeric antibody, IV RTX is also effective in rat models of gestational hypertension [37] and nephropathy [38, 39]. IgM production contributes to pain associated with a tibial mouse fracture and cast immobilization model of CRPS [40, 41].

In the present study, we sought to comparatively assess the role of B cells in neuropathic pain caused by focal PNS trauma in female and male rats. Previously, we reported a gradual increase in serum immunoglobulin (Ig)M autoantibody against MBP(84–104) in female rats with CCI [42]. Further, MBP(84–104) exposure in sciatic nerve activated the B cell receptor pathway preferentially in female rodents [43]. Herein, using female and male rats post-CCI, we demonstrate that effect of IV RTX therapy on neuropathic pain and production of IgM autoantibodies against MBP(84–104) are sexually dimorphic (female-specific).

**Results**

**Sex-specific effect of IV RTX therapy against mechanical hypersensitivity**

CD20 glycoprotein is expressed on the surface of all B cells [33]. Due to sexual dimorphism in immune mechanisms of mechanical hypersensitivity in rodents [21], we here aimed to comparatively assess CD20 levels in female and male mechanical hypersensitivity using a rat sciatic nerve CCI mononeuropathy model. CD20 immunoblotting (Fig. 1A) and immunostaining (Fig. 1B) analyses were carried at 1 week post-CCI, when B cells are known to infiltrate sciatic nerve in males [46]. Spleen from the same animals were used as positive control for CD20 expression (Fig. 1C-D).
In sciatic nerve of both sexes, the expected CD20 monomer (33 kDa) band was present, albeit excessively faint at 50 µg total protein (Fig. 1A) and even 150 µg total protein (data not shown). Noteworthy, there was a CD20-reactive 46 kDa band selectively enriched in male, but not in female, nerves (Fig. 1A) of unknown identity. The CCI nerve displayed small, rounded CD20-positive cells, with no apparent sex difference in their numbers and distribution patterns (Fig. 1B). Control spleen tissues of both sexes showed the expected 33 kDa monomer (Fig. 1C), corresponding to CD20-positive cell clusters (Fig. 1D, shown in male).

B cell targeting by IV RTX, a human-mouse chimeric monoclonal antibody against CD20, attenuates mechanical hypersensitivity in patients with CRPS [35] and anti-MAG IgM neuropathy [36]. IV RTX is efficacious in the rat disease models [37–39]; hence, we here tested the effect of IV RTX therapy on CCI-induced mechanical hypersensitivity in female and male rats (Fig. 2). IVIG, a preparation of heterogeneous polyclonal serum IgG against a broad range of foreign pathogens and autoantigens, was used as control.

Mechanical hypersensitivity was recorded by a stable decline in paw withdrawal thresholds to von Frey stimulation in female and male rats post-CCI (Fig. 2). At day 7 post-CCI, when B cells infiltrate the nerves [46], the rats received bolus IV RTX (10 mg/kg in 10 µl PBS, n = 18 [female n = 9 and male n = 9]), IVIG (10 mg/kg in 10 µl PBS, n = 12 [male n = 6 and female n = 6]) or PBS vehicle (10 µl, n = 12 [male n = 6 and female n = 6]) therapy.

In female rats post-CCI, IV RTX therapy increased mechanical thresholds relative to IV PBS (p = 0.066, ANOVA) (Fig. 3A). In male rats post-CCI, the withdrawal threshold values were variable, resulting in no detectable effect of IV RTX compared with IV PBS treatment (p > 0.05, Fig. 3B). There was no significant change in the withdrawal thresholds (p > 0.05) observed after IVIG therapy relative to IV PBS injection in CCI females (Fig. 2C) or males (Fig. 2D). According to analyses of the area under the curve (AUC) between day 7 and day 16 post-CCI, the effect of IV RTX was significantly different relative to IV PBS in female rats (p = 0.0091, nonparametric Mann-Whitney test, Fig. 2E), but not in male rats (Fig. 2F). The contralateral to CCI hind paws displayed no mechanical hypersensitivity in either sex or treatment (data not shown).

CD20 immunoblotting (Suppl. Figure 1) and immunostaining of sciatic nerve and spleen indicate no change in B cell content upon completion of behavioral testing, at day 17 post-CCI and day 10 post-therapy, regardless of sex or treatment group. We conclude that single bolus IV therapy was not sufficient to deplete B cells and that the analgesia RTX effect in females relates to targeting of B cell activity, not infiltration, in the nerve.

**MBP epitope release in CCI nerve of both sexes**

To elucidate the source of sexual dimorphism in B cell activity post-CCI, we focused the study on the major neural autoantigen, MBP. The AB5864 EDM-Millipore antibody generated against guinea pig MBP(69–86) peptide is used to differentiate demyelinating from intact neural tissues [47]. In peripheral
(sciatic and spinal) nerves of female rats the degraded (d) MBP(69–86) reactivity is present at days 1–27 post-injury [14, 28–30]. Whether the MBP epitope is released in male nerves has not been tested.

Using the AB5864 EDM-Millipore antibody, dMBP immunostaining was carried out in female and male sciatic nerves at days 3, 7 and 27 post-CCI (Fig. 3–4). In nerves of both sexes, the dMBP immunoreactivity was associated with myelin sheath (doughnut structures) and myelinating Schwann cells (crescent structures), particularly preceding demyelination at day 3 post-CCI and after remyelination at day 27 post-CCI (Fig. 3). As expected, the structures were less well defined in the demyelinating day 7 post-CCI nerves (Fig. 3). FluoroMyelin staining highlights dMBP immunoreactivity in perimyelin and myelin ovoid structures in nerves of both sexes (Fig. 4).

**Female-selective serum IgM autoantibody against MBP(84–104) post-CCI**

To assess the serum levels of IgM and IgG anti-MBP autoantibody in female and male rats post-CCI, we employed our ELISA that employed the immobilized MBP(84–104) peptide as bait [42]. We have previously identified MBP(84–104) as the most potent pro-algesic MBP peptide in females rats [14, 31, 43].

In female rats post-CCI, the serum IgM antibody levels increased in a time-dependent manner, peaking at day 28 post-CCI (Fig. 5B, left), as we have shown before [42]. No detectable IgM reactivity above the threshold value was observed with the scrambled (SCR) peptide or the BSA control at any time-point (Fig. 5B, left). This ELISA detects both the IgM and IgG isotypes of anti-MBP(84–104) antibody, but there was no IgG detected in rat serum before or after CCI in either sex (Fig. 4B, right). At day 28 post-CCI relative to the respective naïve control, the specific anti-MBP(84–104) IgM autoantibodies (A450 MBP-SCR) elevated up to 7-fold in female rats relative to the respective control (Fig. 5C). In contrast, there was no anti-MBP(84–104) IgM detected in the serum of male rats, naïve or after CCI (Fig. 5C). We conclude that production and/or circulation of the anti-MBP IgM autoantibodies after PNS injury in rats is prevalent in females.

**Discussion**

After peripheral nerve injury, chronic neuroinflammation contributes to the development of neuropathic pain [10–12]. This process is deemed sexually dimorphic, with T cell-mediated adaptive immune system selectively contributing to mechanical pain hypersensitivity in female rodents with PNS injury [21, 48]. Until the present study, contribution of B cells to sexual dimorphism of neuropathic pain remained not studied. Herein, we provide several independent lines of evidence for the sex-specific B cell activity after focal PNS injury. Thus, (i) B cell targeting using Rituximab immunotherapy resulted in female-specific reduction of mechanical hypersensitivity in rats with sciatic nerve CCI, not observed in males; (ii) exposure of a myelin autoantigen (MBP) epitope in sciatic nerve results in sex-specific regulation of B
cell-related genes; and (iii) serum autoantibodies against MBP is detected in female, but not in male, rats post-CCI.

CD20 targeting using IV Rituximab reduces CCI-induced mechanical hypersensitivity selectively in female rats. We here administered a single bolus IV (tail vein) RTX (40 mg/kg) injection at 1 week post-CCI, the time-point of the nerve B cell infiltration that starts with day 3 post-CCI and gradually increases until at least day 14 post-CCI [46]. We detected no apparent sex differences in the number of the CD20-positive cells as well as the intensity of the CD20 monomer band in CCI nerves. Whether or not he 46 kDa band, selectively enriched in male nerves, represents a post-translationally modified (e.g., glycosylated) CD20 remains to be determined in future analyses of CD20 null tissues and/or CD20 protein neutralization in the future. Regardless of its CD20 specificity, the anti-CD20 detection antibody reactivity of the 46 kDa protein may concern the Rituximab effects in males. As a mouse-human chimeric anti-CD20 antibody, Rituximab neutralizes CD20 in rat models of diabetic nephropathy after IV (tail vein) at 58 mg/kg once weekly for 4 weeks [38], adriamycin-induced nephropathy after IV (tail vein) at 10 mg/kg once weekly [39], and gestational hypertension after continuous infusion at 250 mg/kg [37].

In the nerve and spleen, CD20-reactive cells displayed morphological features of lymphocytes. The single IV Rituximab therapy produced no apparent decline in the cell numbers suggesting its effect on B cell activity in pain processing rather than B cell depletion. The present study was designed to establish, in principle, the sex effect on B cell targeting in painful nerve traumatic injury. Future investigation is required to determine the potential of Rituximab and other B cell modulators as an analgesic strategy in neuropathic pain management. Poor ability of Rituximab to cross neurovascular barriers and a relatively low nerve CD20 level may present a challenge in implementing analgesic Rituximab therapy.

The female-specific effect of B cell targeting in CCI-induced mechanical hypersensitivity agrees with the mounting evidence of female-specific adaptive immune/T cell regulation of mechanical hypersensitivity in rodents with PNS injury [13, 18, 21]. Because an antibody production peaks in several weeks, the sex-specific Rituximab effect at 1 week post-CCI likely relates to non-canonical B cell functions in antigen presentation and T cell activation. Thus, in patients with IgM anti-MAG demyelinating neuropathy, Rituximab therapy mitigates pain and paresthesia through T reg cell activation [36]. Similarly, efficacy of B cell depletion therapy in multiple sclerosis has been attributed to autoantibody-independent reduction of CD4-positive T cell activity [49]. Lack of benefit of IVIG immunotherapy against CCI-induced hypersensitivity and pain in CRPS patients [50] also favors autoantibody-independent B cell functions in pain.

Antigens in focal PNS trauma are not well studied. We have previously established that the autoantigenic MBP epitopes that are concealed from immunosurveillance in healthy nerve are released post-injury by metalloproteases, including MMP-9 [14, 28–31, 42, 51–53]. Several peptides comprising the 87-VVHFF-91 motif of MBP, essential to T cell recognition [54], including MBP(84–104) and MBP(69–86), but not the N-terminal MBP(2–18) peptide (all human sequence and analyzed relative to a scrambled peptide), induce robust mechanical hypersensitivity in female rats [14, 28, 30, 31]. In addition, injection of the rat-specific
MBP(69–86) peptide, matching the endogenously released MBP peptide detected in rat nerve by the AB5864 (EDM-Millipore) antibody, produces mechanical hypersensitivity in rats [28] in support of its auto-antigenic action.

The released dMBP/MBP(69–86) peptide levels and the MBP-degrading protease activity [55] are comparable in female and male CCI nerves, suggesting that sexual dimorphism arises downstream of the MBP epitope action [43]. The equal-dose intraneural MBP(84–104) induces female-specific mechanical hypersensitivity through a vast and sex-specific transcriptional reprogramming in the nerves and the corresponding DRG and spinal cord [43]. Among the major multi-systemic changes induced by intraneural MBP(84–104) is sex-specific changes in lipid-calcium metabolism, T and B cell-related gene activity progressing from the nerve to DRG and spinal cord only in females, but not males [43]. Sex, species, strain, age and other biological variables affecting the activity of immune response genes will likely influence B cell activity in pain.

The serum autoantibodies against MBP(84–104) are detected exclusively in females, starting at 1 week post-CCI, at the time when B cells infiltrate the nerve. While the IgM accumulation post-CCI and intraneural MBP(84–104)-induced pain [43] are both female-specific, no IgM/IgG is detected two weeks after intraneural MBP(84–104) (data not shown), suggesting that nociceptive MBP actions [14, 28, 30, 31, 43] are largely autoantibody-independent. Autoantibodies are thought to contribute to persistent pain states via IgG/Fc receptor (FcR) and the complement system activation on the primary afferents [5–7, 9, 56–59]. While both Ig isotypes can be readily detected by our ELISA methodology [42], IgM alone, but not IgG, anti-MBP(84–104) was detected post-CCI. While the IgM/Fµ receptor is elusive, IgM autoantibodies contribute to pain in the murine tibial fracture and cast immobilization model of CRPS [40, 41]. Accordingly, IgM deficiency mitigates pain behavior, and conversely, IgM injection from wildtype mice resumes the CRPS-like pain [40, 41].

We believe that potential translatability of these findings is high as RTX is FDA approved for use in several diseases. Because MBP84-104 epitope is evolutionarily conserved among mammalian species [30], our ELISA detects anti-MBP(84–104) autoantibodies in female rats post-CCI and in female patients with multiple sclerosis [42]. Based on high structural homology of MBP84-104 with muscarinic M2 acetylcholine receptor, these findings may reflect CRPS and other painful conditions [52]. An anti-MBP IgG, albeit not MBP(84–104)-specific, may benefit nerve repair [60], but whether the autoantibodies have a functional role in pain remains to be determined in long-term models of pain. In unilateral sciatic nerve injury models of neuropathic pain, the site of action is likely ipsilateral of injury and targeted to mechanosensitive myelinated A-fibers. Unmyelinated nociceptive fibers are not a likely site, as intraneural MBP(84–104) does not produce heat hypersensitivity [14, 31, 43].

**Conclusion**

The present study offers strong evidence for sex-specific B cell action after peripheral nerve injury. B cell modulation of mechanical hypersensitivity and production of autoantibodies against a myelin
autoantigen are both female-specific. Whether or not these finding are directly related remains to be
determined. Mechanical hypersensitivity arising due to autoimmune remodeling of myelin on sensory
neuraxis may be amenable to immunotherapeutic intervention, particularly in females.

Methods

Reagents, drugs and antibodies

General reagents were purchased from Sigma-Aldrich and ThermoFisher Scientific. Rituxan (Rituximab,
RTX) was obtained from Genentech. Intravenous immunoglobulin (IVIG) Gamunex-C (immune globulin
injection 10%) was purchased from Grifols. Human intact MBP (18.5 kDa isoform) was purchased from
Meridian Life Science. Based on human MBP sequence (GenBank, AAH08749), MBP(84-104)
(ENPVVHKFNIVTPRTPPPSQ) and scrambled (SCR, EFPHIKVTVTTPNGFPNSPP) peptides, N-terminally
biotinylated and C-terminally amidated, were synthesized by GenScript. Rabbit polyclonal anti-CD20
antibody was obtained from Abcam (ab85809), mouse monoclonal anti-β-actin antibody was obtained
from Sigma (A53166), rabbit anti-degraded (d)MBP antibody, generated against the synthetic peptide
encoding guinea pig MBP(69-86), was obtained from EDM-Millipore (AB5864). FluoroMyelin 488
(F34651) was obtained from Molecular Probes. For ELISA, HRP-conjugated goat anti-rat IgM (3020-05)
and anti-rat IgG (112-035-175, Jackson ImmunoResearch), a 3,3’,5,5’-tetramethylbenzidine substrate
(TMB/E, Surmodics) and BSA (an IgG and protease-free, 30% solution, US Biological) were used.

Animal surgery and sample collection

All animal procedures were performed according to the protocols approved by the Institutional Animal
Care and Use Committee at the Veterans Affairs San Diego Healthcare System, the Public Health Service
Policy on Humane Care and Use of Laboratory Animals and ethical guidelines of the International
Association for the Study of Pain. Sprague-Dawley rats (Envigo, 8-week-old, female and male) were
housed in temperature-controlled cages with a 12-h light–dark cycle and free access to food and water.
The procedures and behavior testing were performed during the light cycle. Under 3% isoflurane
anesthesia, the left common sciatic nerve was exposed at the mid-thigh level with sterile technique. CCI
was produced by tying three loosely constrictive chromic gut ligations around the sciatic nerve [44]. Sham
operation included nerve exposure with no injury. Animals were sacrificed by intraperitoneal Euthasol
(100–150 mg/ml; Virbac Animal Health) after deep isoflurane inhalation anesthesia. Blood, spleen,
sciatic nerve, lumbar (L)5 dorsal root ganglia (DRG) and L1-6 dorsal spinal cord tissues were harvested
snap-frozen in liquid N2 and stored at -80°C for immunoblotting, or following transcardial perfusion in 4%
paraformaldehyde (PFA) in 0.2 M phosphate buffer for immunostaining. For ELISA, 1-2 ml blood aliquots
were collected before CCI and 2, 14 and 28 days post-CCI by cardiac puncture in tubes without anti-
coagulant, allowed to clot for 30 min at ambient temperature, centrifuged (2,000xg; 10 min; 4°C) and the
supernatant (serum) was collected and stored at -80°C.
Drug delivery

At day 7 after CCI, RTX (10 mg/kg, female n=9, male n=9) and IVIG (10 mg/kg, female n=3, male n=4), each diluted in PBS (Steris Labs) vehicle, or PBS vehicle control (female n=3, male n=3), was administered by intravenous (IV) injection through the tail vein in the same volume (10 µl) using a 27-gauge needle.

von Frey testing

Withdrawal threshold to non-noxious mechanical stimuli was assessed by von Frey testing using the Dixon's up-down method [45]. Briefly, animals were acclimated to the Plexiglas compartments with 6-mm wire grid bottom and habituated to the environment for two days prior to baseline testing. Baseline values were established for three consecutive days before surgery and then up to daily between 2 and 16 days post-CCI, as indicated. Mechanical stimuli were applied using von Frey filaments (0.4–15.2 g, Stoelting, Wood Dale, IL, USA) perpendicularly on the plantar area of the hind paw innervated by sciatic nerve. The stimuli were applied for 2 seconds, with a 10-second interval between each stimulus or until the rat was stable from the pain. The responses were recorded as positive if the paw was rapidly withdrawn. The 50% withdrawal threshold was calculated according to the Dixon up-down method. Testing was performed at fixed times between 8:00 a.m. and 2:00 p.m. by experimenters blinded to the treatment groups.

Immunostaining

Tissues (sciatic nerves and spleen) were excised, postfixed in 4% PFA, rinsed in 0.2 M phosphate buffer solution (pH 7.4) for 16-18 h. Tissues were embedded in the optimal cutting temperature compound (Sakura Finetek) following the 15-30% gradient sucrose treatment or paraffin and cut into transverse, 10-µm-thick sections [14, 28, 29, 31]. The paraffin sections were deparaffinized in xylene and rehydrated in ethanol. Non-specific binding was blocked with 5% goat serum for 30 min at ambient temperature, followed by polyclonal rabbit anti-CD20 (ab85809, Abcam, 1:200) or dMBP (AB5864, EDM-Millipore 1:2000) antibody application at 4°C overnight. After rinsing in PBS, the sections were incubated with the goat anti-rabbit Alexa-conjugated secondary antibody (red, ThermoFisher Scientific) for 1 h at ambient temperature. In select frozen sections, FluoroMyelin 488 (F34651, Molecular Probes, 1:500) was applied for 20 minutes at ambient temperature. Slides were mounted in Slowfade Gold anti-fade reagent containing DAPI (4',6-diamidino-2-phenylindole, ThermoFisher Scientific, blue). Staining specificity was confirmed by a primary antibody omission. The images were acquired using All-In-One Fluorescence Microscope BZ-X700 (Keyence, Itasca, IL) and Leica fluorescence microscope.

Immunoblotting
Tissue extracts were prepared in TBS supplemented with 1% Triton X-100, 10% glycerol, 0.1% SDS, 5 mM EDTA, 1 mM PMSF, aprotinin, pepstatin and leupeptin (1 μg/ml each). Insoluble material was removed by centrifugation (14,000×g; 15 min). Extract aliquots (25-50 μg of total protein) were separated by SDS-gel electrophoresis in 15% Tris-glycine gels (Bio-Rad). Separated proteins were transferred onto a PVDF membrane. The membrane was blocked in 5% non-fat milk (Bio-Rad) and incubated for 16-18 h at 4°C with the rabbit polyclonal anti-CD20 antibody (Abcam, ab85809) followed by incubation for 1 h at ambient temperature with the rabbit-specific horseradish peroxidase-conjugated goat secondary antibody (Cell Signaling; 1:10,000 dilution). For loading control, the membranes were re-probed using mouse β-actin antibody (Sigma, A53166). The blots were developed using an enhanced chemiluminescence system (GE Healthcare) or SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific). The membranes were re-probed using a β-actin antibody (loading control). The bands were digitized and quantitated using Image J.

ELISA

Rat serum was subjected to the anti-MBP(84-104) epitope IgG and IgM ELISA [42]. The wells of a 96-well Maxisorp ELISA plate were coated with ExtrAvidin (3 μg/ml in 0.125 ml 15 mm bicarbonate buffer, pH 9.6) or BSA (3 μg/ml, control) for 18 h at 4°C. The wells were blocked for 1 h at 37°C using 1% IgG and protease-free BSA (0.4 ml) in 50 mM Tris-HCl buffer, pH 7.8, containing 1 M NaCl and 0.1% Tween-20 (TBS/T). Incubation with the biotin-labeled MBP(84-104) and SCR peptides (5 μg/ml in 0.1 ml TBS/T-1% BSA, each) continued at 4°C for 16-18 h. Serum samples (diluted 1:50 in 0.1 ml TBS/T-1% BSA) were allowed to bind to the wells for 3 h. The secondary HRP-conjugated goat anti-rat IgG or IgM antibodies (diluted 1:10,000 in 0.1 ml TBS/T-1% BSA, each) were added for 1 h. Six washes (5 min each; 500-700 rpm) in TBS/T at ambient temperature were done after each step. After a wash with H2O, the TMB/E substrate (0.1 ml) was added to the wells. The reaction was stopped using 1 m H2SO4 (0.1 ml) and estimated at A450 using a plate reader. Data represent means ± SE from at least 3 individual experiments performed in triplicate. The A450 values for MBP(84-104) peptide were calculated relative to SCR peptide. The threshold values for MBP (A450 = 0.298) and SCR (A450 = 0.237) peptides were determined as described [42].

Statistical analyses

GraphPad Prism 6.0 software (Synergy Software) was used to conduct two-way repeated measures analysis of variance (ANOVA) or a one-way ANOVA with Bonferroni, Holm-Sidak, or Tukey’s post hoc test, or nonparametric Mann-Whitney rank sum test, as detailed in the figure legends. Data are presented as the mean ± SEM, and differences are considered significant at a P value < 0.05.

Abbreviations
Area under the curve (AUC); CCI, chronic constriction injury; complex regional pain syndrome (CRPS); DAPI, 4',6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; IN, intraneural; IV, intravenous; IVIG, intravenous immunoglobulin; MAG, myelin associated glycoprotein; MBP, myelin basic protein; PNS, peripheral nervous system; RTX, Rituximab; SCR, scrambled peptide; TMB, a 3,3’,5,5’-tetramethylbenzidine substrate;

Declarations

Ethics approval:

All animal procedures were performed according to the PHS Policy on Humane Care and Use of Laboratory Animals and the protocol approved by the Institutional Animal Care and Use Committee at the VA San Diego Healthcare System.

Consent for publication:

Not applicable

Availability of data and material:

Data sharing not applicable to this article. Please contact author for data requests.

Funding:

This research was supported by NIH R01 DE022757 (VIS), NIH Office of Research on Women’s Health (ORWH) Administrative Supplement for Research on Sex/Gender Differences (VIS, AYS, TLY), the U.S. Department of Veterans Affairs 5I01BX000638 (VIS), and the UCSD Academic Senate grant RS283B (VIS).

Acknowledgements:

Not applicable.

Authors’ contributions:

HJL and JD conducted animal procedures, HJL, JD and SKH performed behavioral analysis; HJL, JBL and SKH carried out immunostaining and immunoblotting analyses; AGR developed and performed ELISA; VIS conceived, designed and directed the study; AYS, AVC and TLY contributed to study design,
execution or data analyses; HJL and VIS co-wrote the manuscript; all authors read, edited, and approved the manuscript.

Competing interests:

The authors report no conflict of interest.

References

1. Fillingim, R.B., et al., *Sex, gender, and pain: a review of recent clinical and experimental findings*. J Pain, 2009. 10(5): p. 447-85.

2. Nahin, R.L., *Estimates of pain prevalence and severity in adults: United States, 2012*. J Pain, 2015. 16(8): p. 769-80.

3. Voskuhl, R., *Sex differences in autoimmune diseases*. Biol Sex Differ, 2011. 2(1): p. 1.

4. Whitacre, C.C., S.C. Reingold, and P.A. O’Looney, *A gender gap in autoimmunity*. Science, 1999. 283(5406): p. 1277-8.

5. McMahon, S.B., F. La Russa, and D.L. Bennett, *Crosstalk between the nociceptive and immune systems in host defence and disease*. Nat Rev Neurosci, 2015. 16(7): p. 389-402.

6. Wigerblad, G., et al., *Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism*. Ann Rheum Dis, 2016. 75(4): p. 730-8.

7. Bennett, D.L. and A. Vincent, *Autoimmune pain: an emerging concept*. Neurology, 2012. 79(11): p. 1080-1.

8. Sorkin, L.S., *Antibody activation and immune reactions: potential linkage to pain and neuropathy*. Pain medicine, 2000. 1(4): p. 296-302.

9. Goebel, A., *Autoantibody pain*. Autoimmun Rev, 2016. 15(6): p. 552-7.

10. Myers, R.R. and V.I. Shubayev, *The ology of neuropathy: an integrative review of the role of neuroinflammation and TNF-alpha axonal transport in neuropathic pain*. Journal of the peripheral nervous system: JPNS, 2011. 16(4): p. 277-86.

11. Myers, R.R., W.M. Campana, and V.I. Shubayev, *The role of neuroinflammation in neuropathic pain: mechanisms and therapeutic targets*. Drug Discov Today, 2006. 11(1-2): p. 8-20.

12. Scholz, J. and C.J. Woolf, *The neuropathic pain triad: neurons, immune cells and glia*. Nat Neurosci, 2007. 10(11): p. 1361-8.

13. Moalem, G., K. Xu, and L. Yu, *T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats*. Neuroscience, 2004. 129(3): p. 767-77.

14. Liu, H., et al., *Immunodominant fragments of myelin basic protein initiate T cell-dependent pain*. J Neuroinflamm, 2012. 9: p. 119.
15. Austin, P.J., et al., *Regulatory T cells attenuate neuropathic pain following peripheral nerve injury and experimental autoimmune neuritis.* Pain, 2012. **153**(9): p. 1916-31.

16. Kim, C.F. and G. Moalem-Taylor, *Interleukin-17 contributes to neuroinflammation and neuropathic pain following peripheral nerve injury in mice.* J Pain, 2011. **12**(3): p. 370-83.

17. Liu, H., et al., *The alternatively spliced fibronectin CS1 isoform regulates IL-17A levels and mechanical allodynia after peripheral nerve injury.* J Neuroinflammation, 2015. **12**: p. 158.

18. Kleinschnitz, C., et al., *T cell infiltration after chronic constriction injury of mouse sciatic nerve is associated with interleukin-17 expression.* Exp Neurol, 2006. **200**(2): p. 480-5.

19. Costigan, M., et al., *T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity.* J Neurosci, 2009. **29**(46): p. 14415-22.

20. Draleau, K., et al., *Phenotypic Identification of Spinal Cord-Infiltrating CD4 T Lymphocytes in a Murine Model of Neuropathic Pain.* J Pain Relief, 2014. **Suppl 3**: p. 003.

21. Sorge, R.E., et al., *Different immune cells mediate mechanical pain hypersensitivity in male and female mice.* Nat Neurosci, 2015. **18**(8): p. 1081-3.

22. Boggs, J.M., *Myelin basic protein: a multifunctional protein.* Cell Mol Life Sci, 2006. **63**(17): p. 1945-61.

23. Kadlubowski, M. and R.A. Hughes, *Identification of the neuritogen for experimental allergic neuritis.* Nature, 1979. **277**(5692): p. 140-1.

24. Devor, M., *Ectopic discharge in Abeta afferents as a source of neuropathic pain.* Exp Brain Res, 2009. **196**(1): p. 115-28.

25. Zhu, Y.L., et al., *Early demyelination of primary A-fibers induces a rapid-onset of neuropathic pain in rat.* Neuroscience, 2012. **200**: p. 186-98.

26. Henry, M.A., et al., *Sodium channel expression and localization at demyelinated sites in painful human dental pulp.* J Pain, 2009. **10**(7): p. 750-8.

27. Wu, G., et al., *Degeneration of myelinated efferent fibers induces spontaneous activity in uninjured C-fiber afferents.* J Neurosci, 2002. **22**(17): p. 7746-53.

28. Hong, S., et al., *Reciprocal relationship between membrane type 1 matrix metalloproteinase and the algesic peptides of myelin basic protein contributes to chronic neuropathic pain.* Brain Behav Immun, 2017. **60**: p. 282-292.

29. Kobayashi, H., et al., *MMPs initiate Schwann cell-mediated MBP degradation and mechanical nociception after nerve damage.* Mol Cell Neurosci, 2008. **39**(4): p. 619-27.

30. Chernov, A.V., et al., *Amino acid sequence conservation of the algesic fragment of myelin basic protein is required for its interaction with CDK5 and function in pain.* FEBS J, 2018. **285**(18): p. 3485-3502.

31. Ko, J.S., et al., *Spinal activity of interleukin 6 mediates myelin basic protein-induced allodynia.* Brain Behav Immun, 2016. **56**: p. 378-89.
32. Perera, C.J., et al., *Active immunization with myelin-derived altered peptide ligand reduces mechanical pain hypersensitivity following peripheral nerve injury*. J Neuroinflammation, 2015. 12: p. 28.

33. Weiner, G.J., *Rituximab: mechanism of action*. Semin Hematol, 2010. 47(2): p. 115-23.

34. Kazkaz, H. and D. Isenberg, *Anti B cell therapy (rituximab) in the treatment of autoimmune diseases*. Curr Opin Pharmacol, 2004. 4(4): p. 398-402.

35. Goebel, A. and F. Blaes, *Complex regional pain syndrome, prototype of a novel kind of autoimmune disease*. Autoimmun Rev, 2013. 12(6): p. 682-6.

36. Dalakas, M.C., et al., *Placebo-controlled trial of rituximab in IgM anti-myelin-associated glycoprotein antibody demyelinating neuropathy*. Ann Neurol, 2009. 65(3): p. 286-93.

37. Novotny, S.R., et al., *Activating autoantibodies to the angiotensin II type 1 receptor play an important role in mediating hypertension in response to adoptive transfer of CD4+ T lymphocytes from placental ischemic rats*. Am J Physiol Regul Integr Comp Physiol, 2012. 302(10): p. R1197-201.

38. Li, L., et al., *Rituximab regulates the expression of the Raf kinase inhibitor protein via NF-kappaB in renal tissue of rats with diabetic nephropathy*. Genet Mol Res, 2013. 12(3): p. 2973-81.

39. Takahashi, Y., Y. Ikezumi, and A. Saitoh, *Rituximab protects podocytes and exerts anti-proteinuric effects in rat adriamycin-induced nephropathy independent of B-lymphocytes*. Nephrology (Carlton), 2017. 22(1): p. 49-57.

40. Li, W.W., et al., *Neuropeptide regulation of adaptive immunity in the tibia fracture model of complex regional pain syndrome*. J Neuroinflammation, 2018. 15(1): p. 105.

41. Li, W.W., et al., *Autoimmunity contributes to nociceptive sensitization in a mouse model of complex regional pain syndrome*. Pain, 2014. 155(11): p. 2377-89.

42. Remacle, A.G., et al., *A sensitive and selective ELISA methodology quantifies a demyelination marker in experimental and clinical samples*. J Immunol Methods, 2018. 455: p. 80-87.

43. Chernov, A.V., et al., *A myelin basic protein fragment induces sexually dimorphic transcriptome signatures of neuropathic pain in mice*. J Biol Chem, 2020. 295(31): p. 10807-10821.

44. Bennett, G.J. and Y.K. Xie, *A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man*. Pain, 1988. 33(1): p. 87-107.

45. Chaplan, S.R., et al., *Quantitative assessment of tactile allodynia in the rat paw*. J Neurosci Methods, 1994. 53(1): p. 55-63.

46. Kim, C.F. and G. Moalem-Taylor, *Detailed characterization of neuro-immune responses following neuropathic injury in mice*. Brain Res, 2011. 1405: p. 95-108.

47. Matsuo, A., et al., *Unmasking of an unusual myelin basic protein epitope during the process of myelin degeneration in humans: a potential mechanism for the generation of autoantigens*. Am J Pathol, 1997. 150(4): p. 1253-66.

48. Mogil, J.S., *Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon*. Nat Rev Neurosci, 2012. 13(12): p. 859-66.
49. Stuve, O., et al., *Clinical stabilization and effective B-lymphocyte depletion in the cerebrospinal fluid and peripheral blood of a patient with fulminant relapsing-remitting multiple sclerosis*. Arch Neurol, 2005. **62**(10): p. 1620-3.

50. Goebel, A., et al., *Intravenous immunoglobulin treatment of the complex regional pain syndrome: a randomized trial*. Ann Intern Med, 2010. **152**(3): p. 152-8.

51. Shubayev, V.I., A.Y. Strongin, and T.L. Yaksh, *Role of myelin auto-antigens in pain: a female connection*. Neural Regen Res, 2016. **11**(6): p. 890-1.

52. Shubayev, V.I., A.Y. Strongin, and T.L. Yaksh, *Structural homology of myelin basic protein and muscarinic acetylcholine receptor: Significance in the pathogenesis of complex regional pain syndrome*. Mol Pain, 2018. **14**: p. 1744806918815005.

53. Remacle, A.G., et al., *Interaction of the cryptic fragment of myelin basic protein with mitochondrial voltage-dependent anion-selective channel-1 affects cell energy metabolism*. Biochem J, 2018. **475**(14): p. 2355-2376.

54. Mendz, G.L., W.J. Moore, and R.E. Martenson, *NMR studies of myelin basic protein. XIII. Assignment of histidine residues in rabbit, bovine and porcine proteins*. Biochim Biophys Acta, 1986. **871**(2): p. 156-66.

55. Remacle, A.G., et al., *Acute- and late-phase matrix metalloproteinase (MMP)-9 activity is comparable in female and male rats after peripheral nerve injury*. J Neuroinflammation, 2018. **15**(1): p. 89.

56. Sorkin, L.S., et al., *Antibody directed against GD(2) produces mechanical allodynia, but not thermal hyperalgesia when administered systemically or intrathecally despite its dependence on capsaicin sensitive afferents*. Brain research, 2002. **930**(1-2): p. 67-74.

57. Mifflin, K.A. and B.J. Kerr, *Pain in autoimmune disorders*. J Neurosci Res, 2016.

58. Klein, C.J., et al., *Chronic pain as a manifestation of potassium channel-complex autoimmunity*. Neurology, 2012. **79**(11): p. 1136-44.

59. Xiao, W.H., A.L. Yu, and L.S. Sorkin, *Electrophysiological characteristics of primary afferent fibers after systemic administration of anti-GD2 ganglioside antibody*. Pain, 1997. **69**(1-2): p. 145-51.

60. Vargas, M.E., et al., *Endogenous antibodies promote rapid myelin clearance and effective axon regeneration after nerve injury*. Proc Natl Acad Sci U S A, 2010. **107**(26): p. 11993-8.