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Chronic complex regional pain syndrome patient IgM has pronociceptive effects in the spinal cord, but not skin, of fracture mice

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Abstract

B cells and IgM antibodies mediate nociceptive sensitization in the mouse tibia fracture model of complex regional pain syndrome (CRPS). Previously we observed that injection of serum or IgM from early CRPS patients into muMT fracture mice lacking mature B cells and antibodies was always pronociceptive at 7 days after systemic injection or one hour after intrathecal or intradermal IgM injection, but systemically injected serum from chronic CRPS patients was rarely pronociceptive. The current study found that chronic CRPS patient IgM was always pronociceptive within an hour after intrathecal injection in fracture mice, whereas intradermal injection of IgM into the hindpaw was never pronociceptive. Chronic CRPS IgM had increased binding reactivity to glial fibrillary acidic protein (GFAP) and intrathecal injection of anti-GFAP antibody was pronociceptive in fracture mice. The slow onset of pronociceptive effects after systemic injection of early CRPS patient serum or IgM suggests gradual accumulation of pronociceptive IgM-antigen complexes in skin and spinal cord. Pronociceptive effects chronic CRPS IgM were blocked by a complement C5a receptor antagonist or a global cytokine inhibitor. These data suggest that initially CRPS patients generate pronociceptive IgM autoantibodies directed at skin and spinal cord neoantigens, but over time spinal neoantigens are primarily targeted.

Introduction

Limb fracture is the most common cause of complex regional pain syndrome (CRPS) [1,2] and in over 40 publications we have characterized a rodent tibia fracture model that develops innate and adaptive pronociceptive immune responses resembling those observed in CRPS patients [3,4]. Tibia fracture with 3 weeks cast immobilization induces 5 months of ipsilateral hindlimb allodynia and unweighting, but in fracture muMT mice lacking mature B cells and immunoglobulin the severity and duration of these pain behaviors are markedly diminished [5]. Germinal centers are specialized structures in lymphoid organs where B cells undergo affinity maturation to produce high-affinity antibodies against a specific antigen. After tibia fracture a germinal center reaction develops over a 3 week period in the ipsilateral popliteal lymph node, mirroring the time course for the development of pronociceptive circulating IgM antibodies and IgM immune complex deposition in the injured hindlimb skin, sciatic nerve, and corresponding spinal cord [6,7]. When fracture mouse serum or IgM (but not IgG) is intraperitoneally injected into muMT fracture mice, it triggers increasing fracture hindlimb allodynia and unweighting that peaks at 7 days and resolves by 14 days post injection [5].

Remarkably, when early (<12 months duration) CRPS patient serum or IgM (but not IgG) was intraperitoneally injected into muMT fracture mice, it induced an increase in hindlimb allodynia and unweighting peaking at 7 days and resolving by 14 days after injection, consistent with the half-life of IgM [4]. When tested individually, the serum of every early CRPS patient (20/20 patients) had pronociceptive effects after a single systemic (intraperitoneal) injection in muMT fracture mice, but only 2/20 chronic (>12 month duration) CRPS patient sera were pronociceptive [4]. Significantly, sera from normal subjects (0/20 subjects) and from early orthopedic trauma patients without CRPS (0/15 patients) were not pronociceptive when intraperitoneally injected into muMT fracture mice.

When pooled early CRPS patient IgM (but not IgG) was injected systemically it induced gradual hindlimb allodynia and unweighting peaking at 7 days and resolving by 14 days. Localized injections of early CRPS IgM intradermally into hindpaw or intrathecally at the lumbar level induced nociceptive sensitization in muMT fracture mice that peaked within an hour and resolved by 24 hours [4]. Pooled chronic CRPS patient IgM had pronociceptive effects within an hour after intrathecal injection, but intraplantar injection had no effect on pain behaviors [8].

Using mass spectroscopy on homogenized fracture mouse paw skin or spinal cord run on 2-D gels and probed with fracture mouse or early CRPS patient sera, we previously identified keratin 16, histone 3.2, gamma actin, and alpha enolase as peripherally located potential autoantigens and glial fibrillary acidic protein (GFAP) as a centrally located potential autoantigen [9]. These antigens are located in the cytoplasm and/or nucleus but their epitopes become accessible to autoantibody binding after post-traumatic cellular apoptosis. Previously we observed increased early CRPS patient serum IgM binding to all the peripheral autoantigens, but chronic CRPS sera IgM exhibited increased binding only to gamma actin [4]. Resolved CRPS patient sera IgM autoantigen binding reactivity resembled control sera binding. These results support the hypothesis that early CRPS IgM binds both peripheral and central antigens, while chronic CRPS IgM primarily targets central antigens.

We postulate that the difference in pronociceptive effects between systemic and intrathecal injection of *chronic* CRPS IgM is due to its molecular size (970 kDa) delaying penetration through the blood-spinal cord barrier, with concurrent breakdown of circulating post-injection IgM before pronociceptive spinal cord levels are achieved. Conversely, the permeable capillaries supplying the dermis and nerves of the fracture hindlimb potentially allow circulating *early* CRPS patient IgM access to autoantigens in the dermis and nerves , resulting in pronociceptive immune complex formation and nociceptive sensitization.

Results

Intrathecal injection of individual chronic CRPS patient IgM was always pronociceptive in fracture mice, but intraplantar injections were never pronociceptive

The effects of intrathecally injecting IgM (1ug/5ul, i.t.) from individual chronic (>12 months post injury) CRPS patients (n =10) were evaluated in the muMT fracture mice and all chronic CRPS patient IgM had pronociceptive effects (increased allodynia 10/10 patients and decreased weight bearing 9/10 patients) at 1 hour post injection (Fig. 1A, B). Intrathecal injection of IgM (1ug/5ul, i.t.) from individual control subjects (n=10) was never pronociceptive. Conversely, intradermally injecting IgM (1ug/10ul, i.p.l.) from individual chronic CRPS patients (n = 6) into the fracture hindpaw plantar skin of muMT mice failed to elicit pronociceptive effects and

intradermal injection of IgM from individual control subjects (n = 6) was never pronociceptive (Fig. 1C, D).

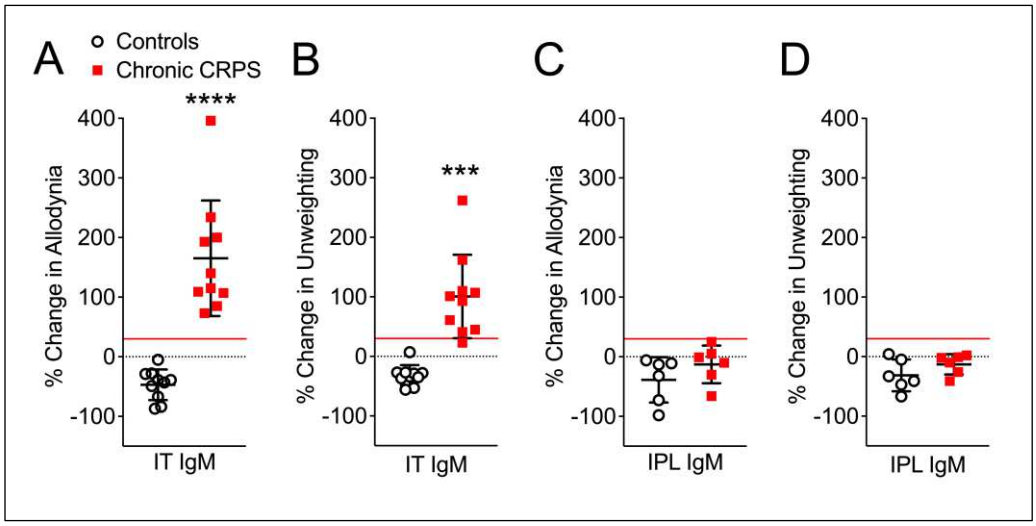


Figure 1. Chronic CRPS patient IgM was always pronociceptive after intrathecal injection in B cell deficient fracture mice, but intraplantar IgM injection had no effect. IgM was purified from serum collected from normal control subjects (Controls, n = 10) and from chronic (> 12 months post injury) CRPS patients (Chronic CRPS, n = 10). At 3 weeks after right tibia fracture and casting in muMT mice lacking mature B cells and IgM, the cast was removed and the next day the mice underwent hindpaw von Frey allodynia (**A**) and unweighting (**B**) testing, then each subject's IgM was intrathecally (i.t.) or intraplantarly (i.p.l.) injected into 3 fracture mice (1ug per mouse) and then the mice were retested at 1 hour post-injection (time point of peak pronociceptive effect). The change in von Frey threshold differences and percent weight-bearing between the pre-injection and the 1-hour post-injection tests were calculated for each mouse and averaged for the 3 mice injected with each subject's IgM. Each point on the scattergram represents an individual patient's average IgM effect on a pronociceptive assay in 3 mice. An increase of greater than 30% (red line) in allodynia or unweighting was considered pronociceptive. All chronic CRPS patients exhibited pronociceptive IgM antibody effects after intrathecal injection. Intrathecal injection of chronic CRPS patient IgM increased ipsilateral hindpaw allodynia (165 ± 31% vs -47 ± 8 % for controls (A) and increased hindlimb unweighting (100 ± 22% vs -39 ± 15% for controls (B). Intraplantar injection of chronic CRPS patient IgM into the fracture limb hindpaw did not increase ipsilateral hindpaw allodynia (-39 ± 15% vs -12 ± 13% for controls (C) or hindlimb unweighting (-32 ± 11% vs -13 ± 7% for

controls (D). An unpaired t-test was used to determine differences between groups. Data are expressed as mean values ± SEM. ***P<0.001, ****P<0.0001 vs Controls. CRPS: complex regional pain syndrome

Increased chronic CRPS patient IgM binding reactivity to GFAP autoantigens

Previously we tested the pronociceptive effects of sera from control subjects (0/20 pronociceptive), early CRPS patients (12/12 pronociceptive), chronic CRPS patients (2/14 pronociceptive), and resolved CRPS patients (0/5 pronociceptive) after intraperitoneal sera injection in muMT fracture mice[4]. When the resolved CRPS patients were initially seen in clinic they met the CRPS Budapest diagnostic criteria, had significant pain symptoms, and their serum and IgM was pronociceptive in muMT fracture mice. When the same patients were followed up in clinic 1-4 years later they no longer met the CRPS Budapest diagnostic criteria, had minimal or no pain, and their sera and IgM was no longer pronociceptive in muMT fracture mice [4,8].

The current experiment utilized the same sera described in our previous study [4] for measuring IgM binding reactivity to full length human GFAP protein. Both early and chronic CRPS patient IgM binding to GFAP was increased relative to control subject IgM immunoreactivity (Fig. 2). Resolved CRPS patient IgM binding to GFAP resembled that of control subject IgM, indicating normalization of GFAP binding reactivity (Fig. 2).

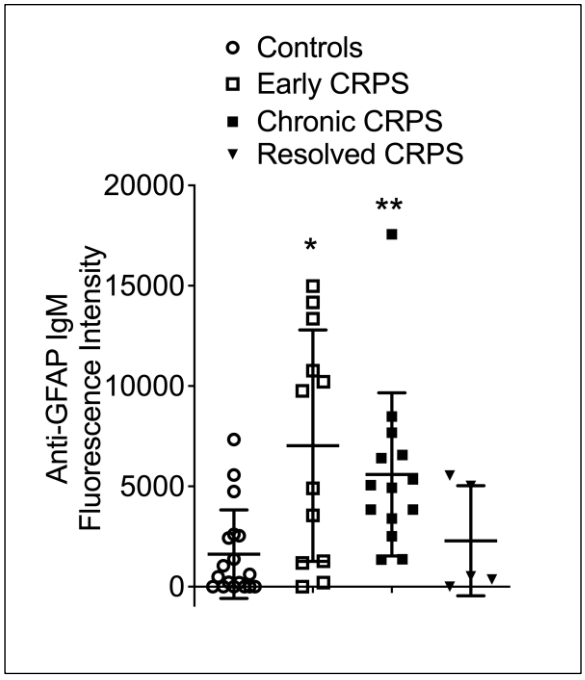


Figure 2. Chronic CRPS patient IgM binding reactivity to GFAP was enhanced.
Sera was collected from normal control subjects (Controls, n=20), early (1-12 months post injury) CRPS patients (Early CRPS, n=12), chronic (> 12 months post injury) CRPS patients (Chronic CRPS, n=14), and patients that initially had CRPS and pronociceptive serum effects in fracture mice, but were re-evaluated at least a year later and at that point had resolved CRPS symptoms and resolved pronociceptive serum effects (Resolved CRPS, n=5). Sera from all four groups were tested with IgM binding dot blot studies using recombinant human GFAP, an intermediate filament protein found in the cytoplasmic cytoskeleton of astrocytes. This potential autoantigen had been identified as a promising autoantigen candidate from our previous liquid chromatography-mass spectrometry studies in fracture mouse skin and spinal cord. Early (7030 ± 1664) and chronic (5596 ± 1086) CRPS patient IgM binding reactivity to GFAP was increased, relative to control subject (1620 ± 520) IgM immunoreactivity. Resolved CRPS patient IgM binding levels (2299 ± 1228) resembled control levels. A one-way analysis of variance was performed followed by a Sidak correction test for post hoc contrasts. Data are expressed as mean values ± SEM. *P<0.05, **P<0.01vs Controls. CRPS: complex regional pain syndrome, GFAP: glial fibrillary acidic protein

Intrathecal injection of anti-GFAP antibody was pronociceptive in fracture mice, but intraplantar injection of anti-GFAP had no effect.

The effects of intrathecally injecting a commercially available mouse polyclonal IgG antibody targeting a broad range of human GFAP epitopes were evaluated in fracture mice. Intrathecal injection of anti-GFAP IgG antibody (1ug/5ul, i.t.) caused increase allodynia and unweighting in muMT fracture mice between 1 and 3 hours after injection, but intrathecally injecting control IgG antibody had no effect (Fig. 3A, B). Conversely, intrathecally injecting anti-GFAP antibody (1ug/10ul, i.p.l.) into nonfracture muMT mice did not elicit pronociceptive changes. Intrathecal anti-GFAP antibody injection in fracture muMT mice had no effect on nociceptive thresholds in the contralateral nonfracture hindlimb (Suppl. Fig. 1). Intradermal injection of anti-GFAP IgG antibody (1ug/10ul, i.p.l.) into the injured limb plantar skin of muMT fracture mice had no pronociceptive effect (Fig. 3C, D). No pronociceptive effects were observed when anti-GFAP IgG antibody (1ug/5ul, i.t.) or GFAP (0.1ug/5ul, i.t.) were individually injected intrathecally into nonfracture muMT mice, but intrathecal injection of an

immune complex formed by anti-GFAP IgG (1ug) antibody bound to GFAP antigen (0.1ug) into muMT nonfracture mice reduced hindpaw von Frey withdrawal thresholds for 6 hours after injection (Fig. 3E).

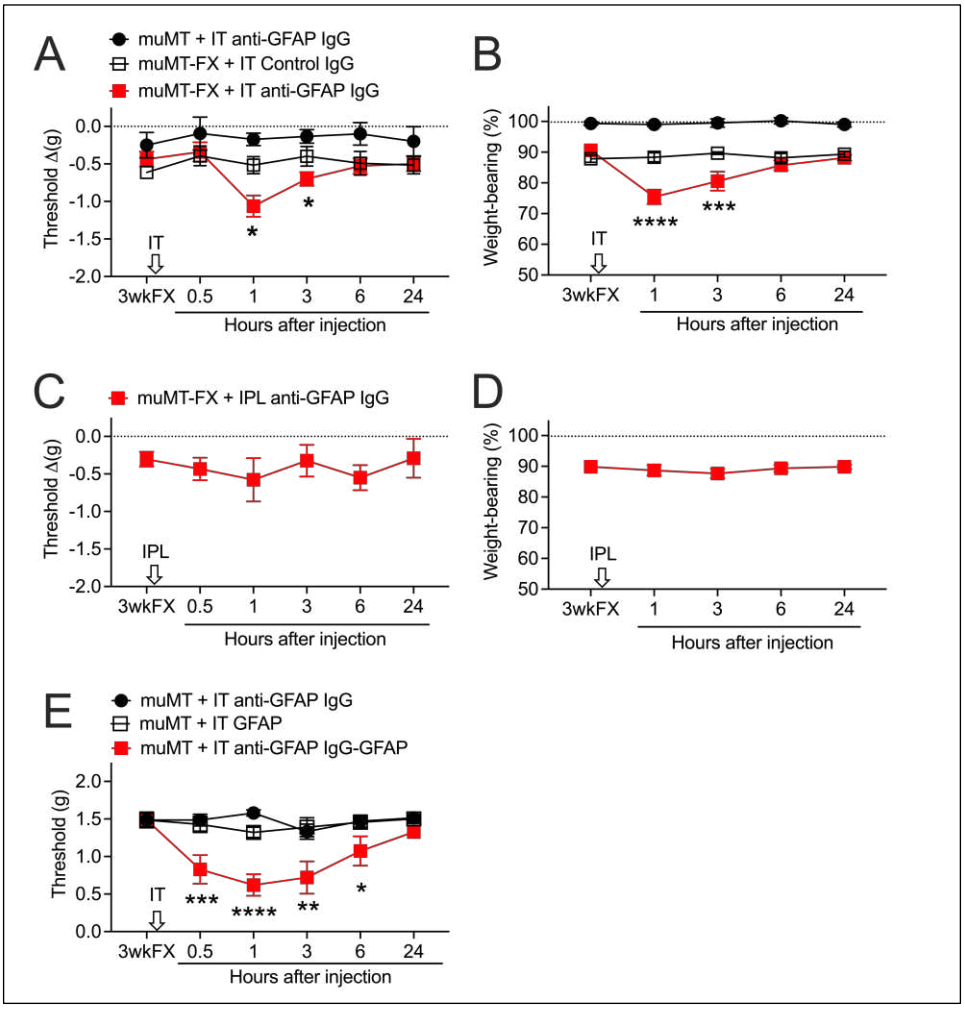


Figure 3. Intrathecal injection of anti-GFAP antibody was pronociceptive in fracture mice, but intraplantar injection of anti-GFAP had no effect. The effects of intrathecally injecting a commercially available mouse polyclonal IgG antibody targeting a broad range of human GFAP epitopes were evaluated in fracture mice. Intrathecal injection of anti-GFAP IgG antibody (1ug/5ul, i.t.) caused increase allodynia (A) and unweighting (B) in muMT fracture mice between 1 and 3 hours after injection, but intrathecally injecting control IgG antibody had no effect. Conversely, intrathecally injecting anti-GFAP antibody (1ug/5ul, i.t.) into nonfracture muMT mice did not elicit pronociceptive changes. Intradermal injection of anti-GFAP IgG antibody (1ug/10ul, i.p.l.) into the injured limb plantar skin of muMT fracture mice had no

pronociceptive effects (C, D). No pronociceptive effects were observed when GFAP (1ug/5ul, i.t.) or anti-GFAP IgG antibody (1ug/5ul, i.t.) were individually injected intrathecally into nonfracture muMT mice, but intrathecal injection of an immune complex formed by anti-GFAP IgG antibody bound to GFAP antigen (1ug/5ul, i.t.) into muMT nonfracture mice reduced hindpaw von Frey withdrawal thresholds for 6 hours after injection (E). A 2-way repeated measures analysis of variance was used to test the effects of each treatment group on the dependent variables over time, using a Sidak correction test for post hoc contrasts. Data are expressed as mean values ± SEM, n = 6-7 mice per cohort. * $P < 0.05$,*** $P < 0.001$, and **** $P < 0.0001$ vs the control IgG treatment group, or in panel E vs the anti-GFAP IgG group. muMT: mice lacking B cells, FX: fracture, IT: intrathecal injection, IPL: intradermal injection into hindpaw plantar skin of the fracture limb, GFAP: glial fibrillary acidic protein

Systemic injection of wildtype fracture mouse IgM or chronic CRPS patient IgM was pronociceptive in fracture mice after osmotic disruption of the blood spinal cord barrier

This experiment used intravenous mannitol injection to disrupt the blood spinal cord barrier. Three-week muMT fracture mice were intraperitoneally injected with 3-week wildtype *fracture mouse serum* (0.5ml, i.p.) and then 2 days later the mice were intravenously injected with either mannitol or saline. Mannitol injected mice developed increased fracture limb von Frey allodynia (Fig. 4A) and unweighting (Fig. 4B) within an hour after mannitol injection, but saline injected mice required 9 days to develop hindpaw nociceptive sensitization, similar to the 7 day latency to peak pronociceptive effect we had previously observed after intraperitoneal injection of early CRPS patient IgM (0.5mg) into muMT fracture mice [4]. Intravenously injected mannitol had no effect on hindpaw von Frey thresholds in the contralateral nonfracture limb of muMT fracture mice previously injected with fracture mouse serum (Suppl. Fig. 2A,B) and had no pronociceptive effects in muMT fracture mice that had not been previously injected with fracture mouse serum (Suppl. Fig.3A,B). Another experiment systemically injected pooled *chronic CRPS patient IgM* (0.5 mg, i.p.) into muMT fracture mice and then 2 days later injected intravenous mannitol or saline. After the mannitol injection the muMT fracture mice developed increased hindlimb allodynia (Fig. 4C) and unweighting (Fig. 4D) within an hour, but saline injected mice required 7 days to develop hindpaw nociceptive sensitization.

The development of nociceptive sensitization 7 days after intraperitoneal chronic CRPS IgM injection in the saline treated mice was unexpected. The dose of systemically injected chronic CRPS patient IgM (0.5mg) corresponds to the amount of IgM in 0.5ml of chronic CRPS patient serum, which is the volume of serum systemically injected in our previous study demonstrating that just 2/20 chronic CRPS patient sera had pronociceptive effects at 7 days post intraperitoneal injection into muMT fracture mice [4]. We postulate that perhaps one of the individual chronic CRPS patient IgM samples included in the pooled chronic CRPS IgM was pronociceptive, resulting is a net pronociceptive effect for the pooled IgM at 7 days after intrathecal injection. We previously have observed that pooled patient IgM samples tend to be more pronociceptive than individual patient IgM samples, perhaps due to a wider array of antibody targeted autoantigens.

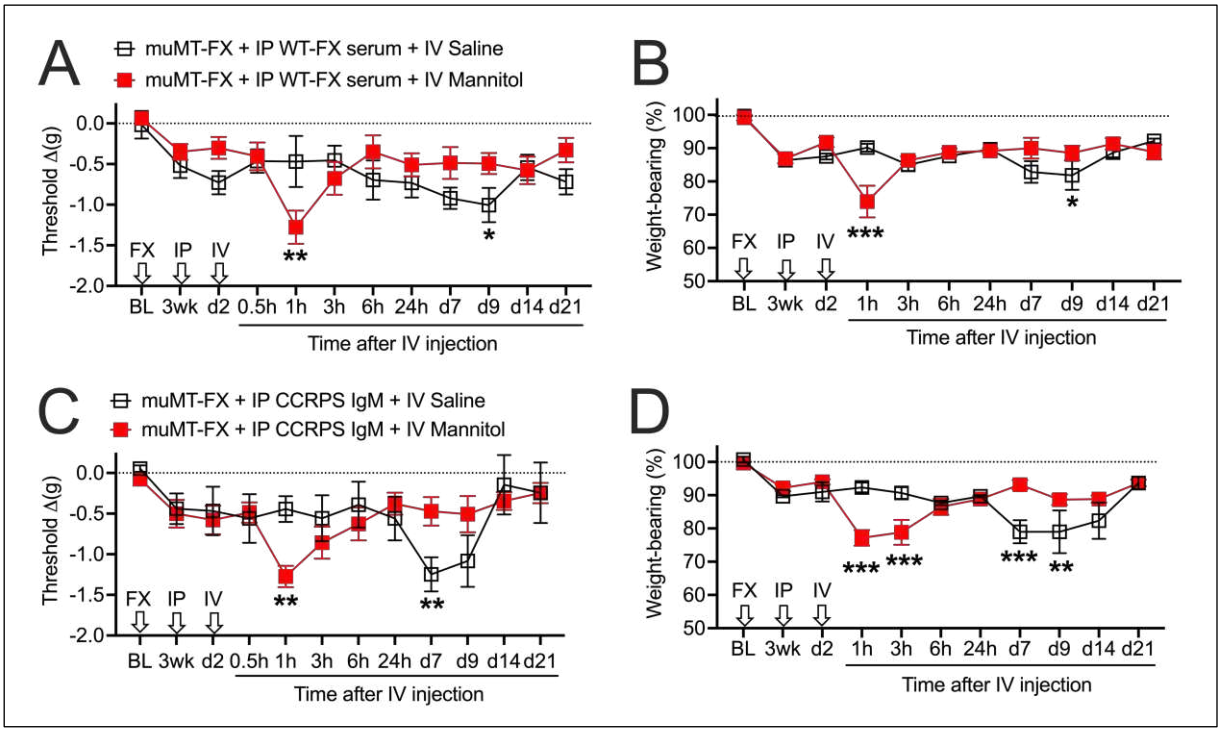


Figure 4. Systemic injection of chronic CRPS patient IgM was pronociceptive in fracture mice after osmotic disruption of the blood spinal cord barrier. This experiment used intravenous mannitol injection to disrupt the blood spinal cord barrier in fracture mice. Three-week post fracture muMT mice were intraperitoneally injected with 3-week wildtype fracture mouse serum (0.5 ml, i.p.) and then 2 days later the mice were intravenously injected with either mannitol (2mg/g body weight, i.v.), or saline. Mannitol injected mice developed increased

fracture limb von Frey allodynia (Fig. 4A) and unweighting (Fig. 4B) within an hour of injection, but saline injected mice required 9 days to develop nociceptive sensitization. When intravenous mannitol was injected into muMT fracture mice that had previously been injected with pooled chronic CRPS patient IgM (0.5 mg, i.p.), the fracture mice developed increased hindlimb allodynia (Fig. 4C) and unweighting (Fig. 4D) within an hour, but saline injected mice required 7 days to develop nociceptive sensitization. A 2-way repeated measures analysis of variance was used to test the effects of each treatment group on the dependent variables over time, using a Sidak correction test for post hoc contrasts. Data are expressed as mean values ± SEM, n = 6 mice per cohort. * $P < 0.05$,** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs the saline control treatment group. WT: wildtype mice, muMT: mice lacking B cells, FX: fracture, IP: intraperitoneal injection, IV: intravenous injection, NS: normal saline, BL: baseline prior to fracture, 3wk: 3 weeks after fracture, CCRPS: chronic complex regional pain syndrome patient

C5a complement spinal signaling mediated the pronociceptive effects of intrathecal IgM in fracture mice

At 3 weeks after right tibia fracture and casting muMT mice lacking B cells and IgM exhibited attenuated unilateral hindpaw von Frey allodynia (withdrawal thresholds reduced by 0.6g) and unweighting (weight bearing reduced by 15%) the day after cast removal (Fig. 5A, B). Intrathecal injection of pooled chronic CRPS patient IgM (1ug/5ul, i.t.) into the 3-week post fracture muMT mice caused an increase in fracture limb hindpaw von Frey allodynia and unweighting between 1 and 3 hours post injection, resolving by 6 hours (Fig. 5A, B). The peak magnitude (1 hour post injection) of IgM induced allodynia and unweighting in muMT fracture mice resembled the magnitude of nociceptive sensitization previously observed in 3-week post fracture wildtype mice [5]. Co-injecting the C5a receptor antagonist PMX53 (200ng) with the intrathecal injection of chronic CRPS patient IgM completely blocked IgM pronociceptive effects. Intrathecal injection of pooled chronic CRPS patient IgM (1ug/5ul, i.t.) into 3-week post fracture muMT mice increased hindpaw von Frey allodynia in the fracture limb at 1-hour post-injection versus 3-week fracture thresholds, but had no pronociceptive effect in the contralateral nonfractured hindlimb (Suppl. Fig. 4A). When chronic CRPS patient IgM was intrathecally co-injected with PMX53 into muMT 3-week post fracture mice there was no pronociceptive effect in either the fracture limb or contralateral nonfracture limb (Suppl. Fig. 4B). As expected, when

pooled control subject IgM was intrathecally injected into fracture muMT mice it had no effect on pain behaviors (Fig. 5C, D) and co-injecting PMX53 with control subject IgM had no pronociceptive or analgesic effects.

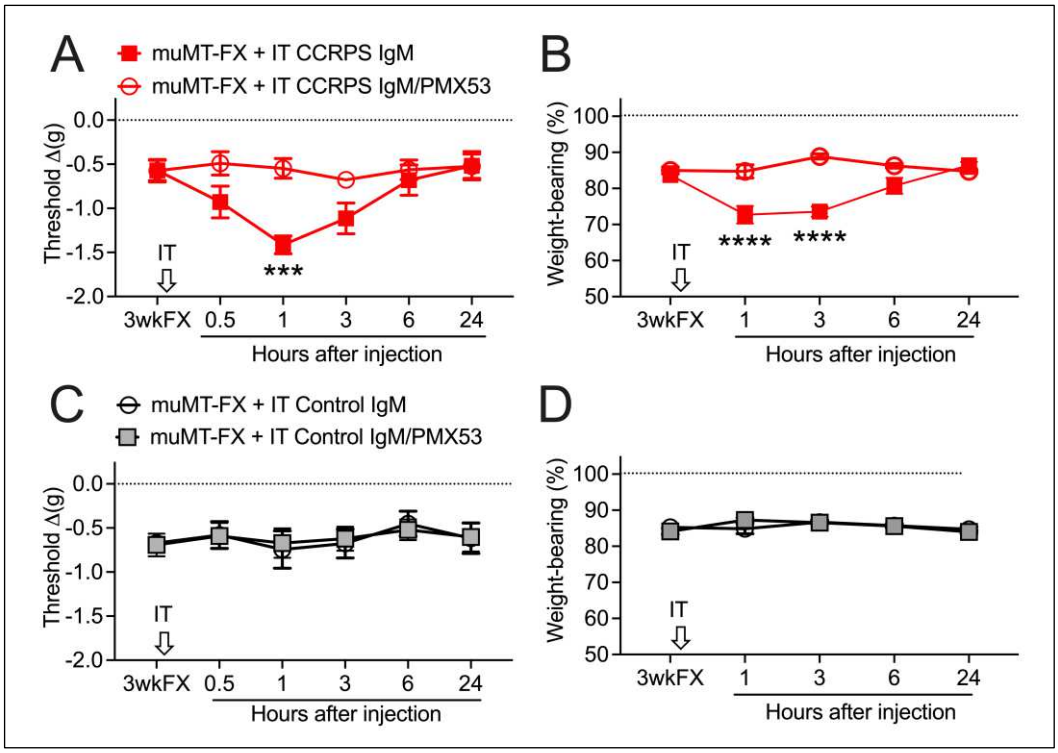


Figure 5. C5a complement spinal signaling mediated the pronociceptive effects of intrathecal chronic CRPS patient IgM in fracture mice. Intrathecal injection of pooled chronic CRPS patient IgM (1ug/5ul, i.t.) into the 3-week post fracture muMT mice caused an increase in fracture limb hindpaw von Frey allodynia (A) and unweighting (B) between 1 and 3 hours post injection, resolving by 6 hours. When the C5a receptor antagonist PMX53 (200ng, i.t.) was intrathecally co-injected with chronic CRPS patient IgM, it completely blocked antibody mediated pronociceptive effects. When pooled control subject IgM was intrathecally injected into fracture muMT mice it had no effect on pain behaviors (**C, D**) and co-injecting PMX53 with control subject IgM had no pronociceptive or analgesic effects. A 2-way repeated measures analysis of variance was used to test the effects of each treatment group on the dependent variables over time, using a Sidak correction test for post hoc contrasts. Data are expressed as mean values ± SEM, n = 7 mice per cohort. *** $P < 0.001$, and **** $P < 0.0001$ vs the PMX53 treatment group. muMT: mice lacking B cells, FX: fracture, IT: intrathecal injection 3wk: 3

weeks after fracture, CCRPS: chronic complex regional pain syndrome patient, control: normal subject, PMX53: C5a complement receptor antagonist

C5a complement signaling mediated upregulated spinal cytokine mRNA expression after intrathecal IgM injection in fracture mice

Intrathecal injection of pooled chronic CRPS patient IgM (1ug/5ul, i.t.) into 3-week post right tibia fracture muMT mice caused increased mRNA expression of TNF and IL-1 cytokines in the right side of the lumbar spine at 1 hour post injection, compared to mice injected with control subject IgM (Fig. 6). Co-injecting the C5a receptor antagonist PMX53 (200ng) with the intrathecal injection of chronic CRPS patient IgM completely blocked IgM stimulation of cytokine expression in the spinal cord.

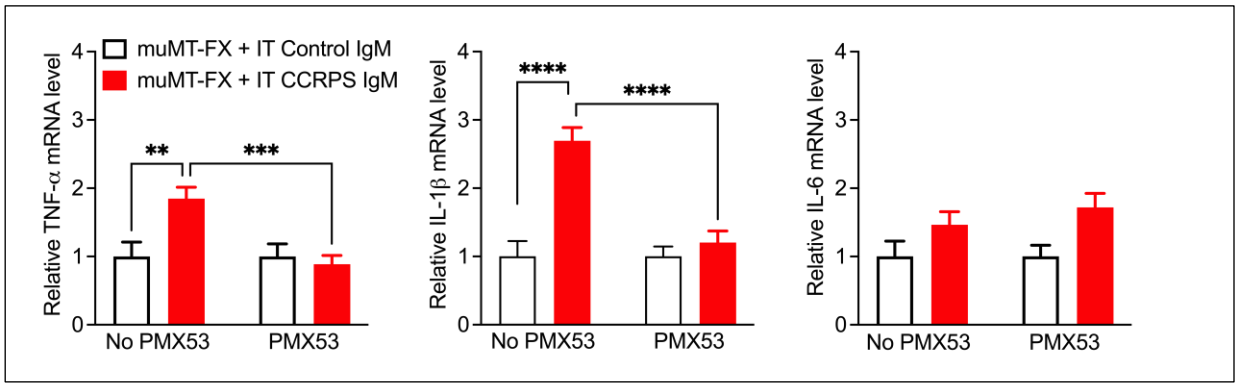


Figure 6. C5a complement signaling mediated spinal cytokine stimulation after intrathecal injection of chronic CRPS patient IgM in fracture mice. At 1 hour post injection the intrathecal injection of pooled chronic CRPS patient IgM (1ug/5ul, i.t.) into 3-week muMT fracture mice caused increased mRNA expression of TNF and IL-1 cytokines in the lumbar spine, compared to mice injected with control subject IgM. Co-injecting the C5a receptor antagonist PMX53 (200ng) with the intrathecal injection of chronic CRPS patient IgM completely blocked antibody stimulation of cytokine expression in the spinal cord. A one-way repeated measures analysis of variance was used to test the effects of each treatment group on the dependent variables, using a Sidak correction test for post hoc contrasts. Data are expressed as mean values ± SEM, n = 7 mice per cohort. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs the control IgM group or vs the no PMX53 treatment group. muMT: mice lacking B cells, FX:

fracture, IT: intrathecal injection, CCRPS: chronic complex regional pain syndrome patient, control: normal subject, PMX53: C5a complement receptor antagonist

TNF and IL-1 protein levels were upregulated in the lumbar spinal cords of fracture muMT mice

TNF and IL-1 protein levels were quantified in lumbar spinal cord samples collected from muMT nonfracture control mice and from 3-week post fracture muMT mice. The fracture mouse spinal cord samples were collected 1 hour after intrathecal injection of either control subject IgM or chronic CRPS patient IgM. Right tibia fracture increased TNF and IL-1 protein levels in the right lumbar spinal cord at 3 weeks post fracture (Suppl. Fig. 5), consistent with our previous observations of neuropeptide dependent upregulation of both mRNA and protein expression of TNF and IL-1 pronociceptive cytokines in the lumbar spinal cord of tibia fracture mice and rats [10,11].

At 1 hour after intrathecal injection of chronic CRPS patient IgM there was no increase in spinal cord TNF and IL-1 protein levels, vs control subject IgM injection (Suppl. Fig. 5). These results are consistent with our experimental hypothesis (Fig. 7) that intrathecal injection of chronic CRPS patient pronociceptive IgM antibodies preferentially target apoptotic neoantigens in the spinal cord, thus forming immune complexes capable of activating the C1 complex that initiate the classical complement pathway, thus generating C5a complement components capable of binding the C5aR receptors on dermal macrophages and spinal microglia to induce the production and secretion of pronociceptive TNF and IL-1 cytokines [12-14]. Intrathecal injection of chronic CRPS IgM into fracture muMT mice would not be expected to alter lumbar spinal cord cytokine protein levels within an hour post injection, but could activate C5a complement within minutes of injection and stimulate microglia release of pronociceptive TNF and IL-1 protein in less than an hour [15-17].

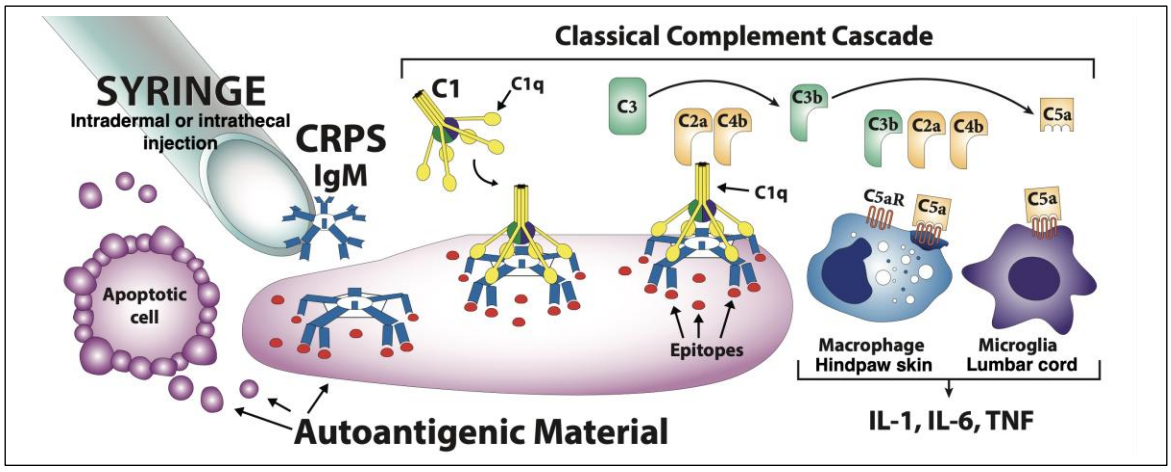


Figure 7. Overview of the hypothesis that autoimmune signaling pathways mediate the pronociceptive effects early and chronic CRPS patient IgM in muMT fracture mice.

Immunoglobulin M predominantly serves as a potent activator of the classical complement pathway. When early CRPS patient IgM is injected intradermally or intrathecally into 3-week post fracture muMT mice lacking mature B cells and antibodies, the IgM antibody attaches to autoantigenic material released from apoptotic cells in the skin or spinal cord to form immune complexes. Similarly, intrathecal injection of chronic CRPS patient pronociceptive IgM antibodies preferentially target apoptotic neoantigens in the spinal cord. When pentameric IgM binds to antigenic material larger than itself it can induce the conformational changes required to allow Fc fragments to bind with C1q complement. C1q, together with C1r and C1s, form to activate the C1 complex that initiates the classical complement pathway, thus generating the C5a complement components capable of activating the C5aR receptors on dermal macrophages and spinal microglia to induce the production and secretion of pronociceptive IL-1, IL-6, and TNF cytokines.[12-14] C5b-9 complement levels are elevated in fracture limb skin and nerve [18] and dermal levels of C5b-9 in hindpaw skin are increased after intradermal injection of early CRPS patient IgM, [8] consistent with activation of the complement cascade. Dermal macrophages and spinal microglia are activated after fracture with increased gene and protein expression of pronociceptive IL-1, IL-6, and TNF cytokines in the hindpaw skin and lumbar spinal cord contributing to allodynia and unweighting in the injured limb.[3,10,19] When IL-1, IL-6, or TNF are injected intradermally into hindpaw skin they each individually evoked mechanical allodynia,[20,21] consistent with literature demonstrating rapid direct local sensitizing effects on sensory nociceptive neurons and long lasting sensitizing effects in the

dorsal horn neurons.[22-24] In summary, these data support the hypothesis that nociceptive sensitization will occur when an immune complex forms in a confined anatomical compartment containing immune cells capable to producing pronociceptive cytokines in response to C5a complement stimulation.

***Spinal cytokine signaling mediated intrathecal IgM pronociceptive effects in fracture** mice*

At 3 weeks after right tibia fracture and casting muMT mice lacking B cells and IgM exhibited attenuated unilateral hindpaw von Frey allodynia and unweighting the day after cast removal (Fig. 8A, B). After behavioral testing the mice were treated with the global cytokine inhibitor pentoxifylline for 2 days. Pentoxifylline treatment (200mg/kg by gavage once daily for 2 days) in fracture mice had no effect on nociceptive thresholds. Intrathecal injection of pooled chronic CRPS patient IgM into pentoxifylline pretreated fracture mice had no pronociceptive effects, but IgM injection into water pretreated fracture mice caused increased allodynia and unweighting in the fracture limb. Intrathecal injection of pooled chronic CRPS patient IgM (1ug/5ul, i.t.) into water pretreated 3-week post fracture muMT mice increased hindpaw von Frey allodynia in the fracture limb at 1 hour post injection, versus the 3-week fracture threshold, and had no pronociceptive effect in the contralateral nonfractured hindlimb (Suppl. Fig. 6A). Intrathecal injection of chronic CRPS IgM into pentoxifylline pretreated fracture mice had no pronociceptive effects in either limb (Suppl. Fig. 6B).

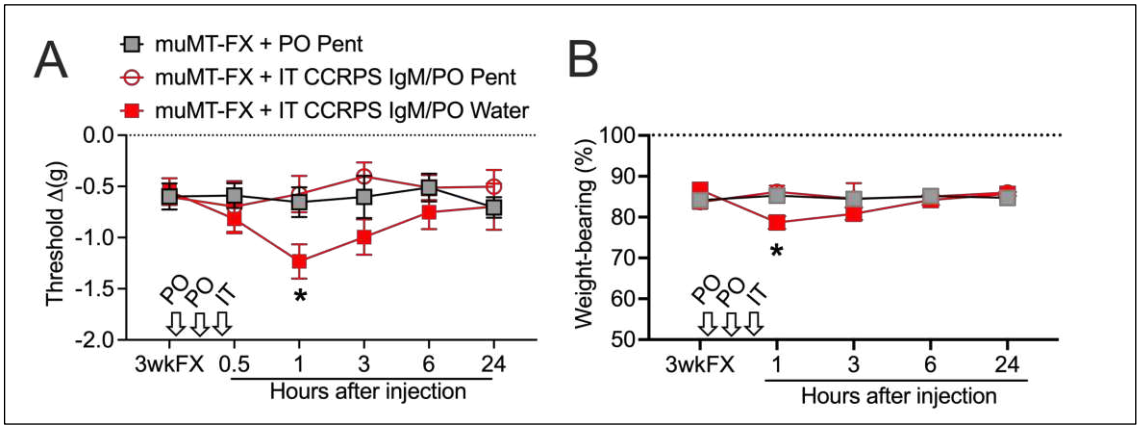


Figure 8. Spinal cytokine signaling mediated intrathecal chronic CRPS patient IgM pronociceptive effects in fracture mice. At 3 weeks after right tibia fracture and casting muMT mice lacking B cells and IgM exhibited attenuated unilateral hindpaw von Frey allodynia (A) and unweighting (B) the day after cast removal. After behavioral testing the mice were treated with the global cytokine inhibitor pentoxifylline for 2 days. Pentoxifylline treatment (200mg/kg by

gavage once daily for 2 days) in fracture mice had no effect on nociceptive thresholds. Intrathecal injection of pooled chronic CRPS patient IgM into pentoxifylline pretreated fracture mice had no pronociceptive effects, but IgM injection into water pretreated fracture mice caused increased allodynia and unweighting in the fracture limb. A 2-way repeated measures analysis of variance was used to test the effects of each treatment group on the dependent variables over time, using a Sidak correction test for post hoc contrasts. Data are expressed as mean values ± SEM, n = 6 mice per cohort. * $P < 0.05$ vs the water treatment group. muMT: mice lacking B cells, FX: fracture, IT: intrathecal injection, PO: orally administered, 3wk: 3 weeks after fracture, CCRPS: chronic complex regional pain syndrome patient, Pent: pentoxifylline, a global cytokine inhibitor

Discussion

This study demonstrated that: 1) every chronic CRPS patient IgM tested (10/10 patients) was pronociceptive when intrathecally injected into muMT fracture mice, and control subject IgM (10/10 subjects) was never pronociceptive, 2) intradermal injection of chronic CRPS IgM (6/6 patients) was never pronociceptive, 3) both early and chronic CRPS patient IgM exhibited increased binding affinity for GFAP and intrathecal injection of anti-GFAP antibodies into muMT fracture mice induced nociceptive sensitization, 4) osmotic disruption of the blood-spinal cord barrier caused rapid nociceptive sensitization in fracture muMT mice previously systemically injected with chronic CRPS IgM , 5) co-injecting a C5a receptor antagonist with an intrathecal chronic CRPS IgM injection prevented nociceptive sensitization, 6) intrathecal injection of chronic CRPS IgM upregulated the expression of TNF and IL-1 cytokines in the lumbar spine of muMT fracture mice and co-injection of a C5a receptor antagonist blocked this effect, 7) pretreatment of muMT fracture mice with a global cytokine inhibitor blocked the pronociceptive effects of intrathecally injected chronic CRPS IgM. Collectively, these results further the hypothesis that pronociceptive autoimmune responses contribute to early and chronic CRPS pain.

Previously we observed that systemic (intraperitoneal) injection of serum from early (< 12 month duration) CRPS patients to muMT fracture mice always (20/20 patients) increased allodynia and unweighting in the injured limb, but this pronociceptive effect developed slowly over a 1 week period [4]. In contrast, systemic injection of serum from chronic (>12 month

duration) CRPS patients to fracture mice was rarely (2/20 patients) pronociceptive. The current study examined whether these incongruent results could be explained by; a) differences in the array of autoantigens targeted by early and chronic CRPS antibodies, and b) differences in the post-injection latency for the development of IgM pronociceptive effects in the skin and spinal cord of muMT fracture mice lacking B cells and antibodies.

Both early and chronic CRPS patient IgM binding reactivity to GFAP was increased (Fig. 2). Resolved CRPS patient IgM no longer exhibited increased GFAP binding affinity. Intrathecal injection of anti-GFAP antibody exacerbated hindpaw allodynia and unweighting in the fracture limb of muMT mice, but intradermal injection of anti-GFAP antibody into the fracture limb plantar skin had no effect (Fig. 3). Collectively, these data suggest that early CRPS patient pronociceptive IgM antibodies bind to peripheral skin neoantigens and central spinal neoantigens, while chronic CRPS patient pronociceptive IgM antibodies primarily target spinal neoantigens.

Pronociceptive antibodies have no effect until they bind to their targeted neoantigens and form pronociceptive immune complexes. When IgM antibodies from wildtype fracture mice or early CRPS patients are injected systemically or intrathecally in muMT *nonfracture control mice* they do not affect nociceptive thresholds. Similarly, when pronociceptive IgM antibodies from wildtype fracture mice or CRPS patients are injected systemically or intrathecally in muMT *fracture mice* they have pronociceptive effects in the fracture hindlimb but do not alter nociceptive thresholds in the contralateral nonfracture limb or forelimbs. When anti-GFAP IgG antibodies were injected intrathecally in muMT *fracture mice* they caused nociceptive sensitization in the fracture limb only, but not in the contralateral limb (Suppl Fig.1). Intrathecally injecting anti-GFAP IgG antibodies or GFAP antigen into *nonfracture control mice* had no effect on nociceptive thresholds, but intrathecal injection of anti-GFAP IgG antibody bound to GFAP protein reduced nociceptive thresholds in the bilateral hindlimbs of muMT *nonfracture control mice* (Fig.3E).

Intraperitoneal injection of early CRPS patient IgM into fracture muMT mice causes gradual nociceptive sensitization in the injured hindlimb that peaks after a week,[4] but intradermal injection of early CRPS IgM into the plantar skin of the fracture limb has a rapid pronociceptive effect, peaking at 1 hour post injection [8]. Similarly, the intrathecal injection of early CRPS patient IgM autoantibodies evoke pronociceptive effects within an hour [8].

These treatments have no effect in control nonfracture mice. We postulated that the delay in nociceptive sensitization after systemic injection of IgM reflects the time required for injected circulating IgM antibodies to accumulate to pronociceptive levels in the hindpaw plantar skin or spinal cord of fracture muMT mice. If the latency for systemically injected IgM to permeate the spinal cord is greater than the latency for its accumulation in hindpaw skin, it would explain why early CRPS patient serum systemic injections were pronociceptive, but chronic CRPS patient serum systemic injections lacked effect. Approximately 14% of circulating IgM is metabolized daily, so if after a single systemic injection of chronic CRPS patient serum the spinal accumulation of pronociceptive IgM develops too slowly then IgM levels will never reach the threshold for nociceptive sensitization. These data suggest that early CRPS patient serum and IgM injections are pronociceptive because they target numerous peripheral autoantigens in the fracture paw skin [4].

To experimentally address the question of *systemic* versus *local* IgM injection latency differences we used intravenous mannitol to induce temporary osmotic disruption of the endothelial tight gap junctions in fracture muMT mice that had previously been intraperitoneally injected with wildtype fracture mouse serum 2 days prior to mannitol treatment. Mannitol treatment caused a rapid increase in hindpaw allodynia and unweighting in the serum injected fracture mice within an hour post injection (Fig. 4A, B), results supporting the premise that the differences in onset latency for the pronociceptive effects of *systemic* versus *localized* injected IgM are due to the delayed passage of systemically circulating injected IgM through the vascular gap junctions of the hindpaw dermis and/or lumbar spinal cord of the fracture mice. Mannitol treatment in fracture mice systemically injected with chronic CRPS patient IgM caused rapid nociceptive sensitization, presumably due to pronociceptive IgM autoantibodies crossing the blood-spinal cord barrier and accumulating in the spinal cord tissues (Fig. 4C, D).

Another fundamental question arising from this work is regarding the signaling mechanisms mediating the spinal pronociceptive effects of chronic CRPS patient IgM autoantibodies. Both early and chronic CRPS patient IgM antibody effects are primarily mediated by complement activation in the mouse fracture model of CRPS (Fig. 7). Previously we used early CRPS patient IgM to investigate peripheral and central neuroimmune signaling mechanisms mediating autoantibody induced nociceptive sensitization[8]. Fracture in wildtype mice caused a dramatic increase in C5aR expressing macrophages in the dermis of the injured

limb skin and C5aR expressing microglia in lumbar spinal cord. Injecting early CRPS IgM intradermally or intrathecally in muMT fracture mice caused nociceptive sensitization between 1 and 3 hours post inject and co-injecting IgM with PMX53, a C5aR antagonist, blocked this sensitization[8]. Furthermore, when fracture muMT mice were pretreated with pentoxifylline, a global cytokine inhibitor, the pronociceptive effects of intraplantar or intrathecal injection of early CRPS IgM into muMT fracture mice were completely blocked. Intraplantar injection of early CRPS IgM in muMT fracture mice caused increased C1q, TNF, IL-1, and IL-6 expression in the injured hindlimb skin and co-injecting C5aR antagonist PMX53 with early CRPS IgM blocked this effect[8]. Similarly, intrathecal injection of early CRPS IgM into muMT fracture mice caused increased TNF and IL-1 expression in the lumbar spinal cord and co-injecting PMX53 with early CRPS IgM blocked this effect.

The immune signaling mechanisms mediating the spinal pronociceptive effects of chronic CRPS patient IgM autoantibodies are identical to those mediating the spinal pronociceptive effects of early CRPS patient IgM antibodies[8]. Intrathecal injection of chronic CRPS IgM into muMT fracture mice induced nociceptive sensitization within an hour and this pronociceptive effect was blocked by co-injection with the C5aR antagonist PMX53 (Fig. 5). Intrathecal chronic IgM injection caused nociceptive sensitization and increased spinal expression of TNF and IL-1 inflammatory cytokines at 1 hour post injection, and these effects were completely blocked by co-injection of PMX53 (Figs. 6, 8).

In summary, one fundamental proof of autoimmune disease is the demonstration of pathogenic autoantibodies. The "two hit" passive transfer fracture CRPS mouse model requires the post traumatic development of both circulating autoantibodies and cognate self-antigens in the injured limb and corresponding innervating spinal cord. This study demonstrated that IgM from chronic CRPS patients was always pronociceptive when injected intrathecally, but not intradermally, into muMT fracture mice lacking B cells and antibodies, while IgM from normal control subjects was never pronociceptive. When chronic CRPS patient IgM was systemically injected into muMT fracture mice and the blood-spinal cord barrier was transiently osmotically disrupted, similarly rapid nociceptive sensitization developed. Collectively these results suggest that within a year after injury CRPS patients no longer produce pronociceptive IgM antibodies primarily targeting *peripheral* neoantigens, but instead express IgM antibodies targeting *spinal* neoantigens. IgM is a 970kD antibody with a 5-day serum half-life that doesn't readily enter

tissues, suggesting that the lack of pronociceptive effects with chronic CRPS patient sera are attributable to delayed IgM penetration through the blood-spinal cord barrier with concurrent breakdown of circulating IgM before pronociceptive spinal cord IgM levels were achieved.

We postulate that chronic CRPS peripheral limb pain is perpetuated by autoantibody sensitization of spinal cord afferent neurons, which potentially explains the persistent pain symptoms observed in most CRPS patients undergoing amputation of the affected limb [25-27]. The current study advances our understanding of CRPS autoimmune mechanisms and potentially provides a neuroimmune basis for more effective target specific treatments for this debilitating chronic pain state.

Methods

Subjects and clinical data collection

The study protocols were approved by the respective local institutional review boards in accordance with the Declaration of the World Medical Association. All subjects were enrolled after giving written informed consent. Serum was collected from two cohorts of subjects; 1) chronic CRPS patients, and 2) age and sex matched normal control subjects. Chronic (>12 months duration) CRPS patient sera were from a cohort of well characterized patients who had participated in a clinical trial (ethical reference for the trial and blood donation 12/EE/0164) [28]. Inclusion criteria for the chronic CRPS cohort are described in detail elsewhere [28]; in brief, patients met the Budapest clinical scientific criteria for CRPS [29] at the time of serum collection and a duration of CRPS greater than 12 months post-injury. The chronic CRPS patients (n = 10) included 8 females and 2 males, their average age was 41 ± 3 years, their average duration of CRPS since onset of the original injury was 2.6 ± 0.4 years, and their average numerical pain scale at the time of serum collection was 7.4 ± 0.2 (0-10, no pain to the worst pain possible). The normal control subject sera were collected by a commercial company (BioreclamationIVT, Westbury, NY) using our serum processing protocol and all control subjects were tested negative for HIV and HCV antibodies and non-reactive for HBSAG, HIV-1 RNA, HCV RNA, HBV DNA, and STS. The control subjects (n = 10) included 9 females and 1 male, and their average age was 39.2 ± 3.6 years. There was no patient or public involvement in planning this study.

Animals

The experimental protocols used in this study were approved by the Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee (Palo Alto, CA, USA) and followed the animal subjects guidelines of the International Association for the Study of Pain and the ARRIVE guidelines. Three-month-old male muMT mice lacking mature B cells and immunoglobulin, on a C57BL/6J congenic background (#002288, Jackson Laboratory, Bar Harbor, ME), were used in these experiments. Three-month-old male C57BL/6J wildtype mice (#000664, Jackson Laboratory, Bar Harbor, ME) were used as controls. The animals were housed 4 per group in the same room, under pathogen-free conditions with soft bedding, and were given food and water *ad libitum*, with a 12:12 light:dark cycle. During the experimental period the animals were fed Teklad lab rodent diet 2018 (Harlan Laboratories, Indianapolis, IN), which contains 1.0% calcium, 0.7% phosphorus, and 1.5 IU/g vitamin D3. Data collection was conducted blind to group and treatment assignment. There was no randomized assignment of mice to specific treatments and all mice were acclimatized for a minimum of one week in our veterinary facility. At the completion of each experiment the mice were euthanized by carbon dioxide inhalation followed by cervical dislocation. A total 318 mice were used for this research project.

Only male muMT mice were used in this study based on our prior investigations evaluating potential sex differences in nociceptive immune responses in the tibia fracture mouse model [30,31]. No significant differences were observed between male and female pronociceptive innate and adaptive immune responses to fracture, other than the lack of intrathecal minocycline analgesia in the female wildtype fracture mice, but intrathecal minocycline was analgesic in female muMT fracture mice, suggesting neuroimmune plasticity in female mice lacking adaptive immunity. Long-lasting immune changes develop in the fracture limb and corresponding spinal cord of both male and female wildtype mice, including upregulated neuropeptide and cytokine signaling, microglial activation, and pronociceptive autoimmunity. The chronic CRPS patient IgM antibodies tested in this study were obtained primarily from female patients, but the IgM antibodies obtained from every individual female and male chronic CRPS patient evaluated in the current study exhibited pronociceptive effects after intrathecal injection into muMT fracture mice.

Surgery

The fracture model was performed in 3-month-old male mice as previously described [32]. Under isoflurane anesthesia a hemostat was used to make a closed fracture of the right tibia just distal to the middle of the tibia. The hindlimb was then wrapped in casting tape (Delta-Lite, BSN Medical, Hamburg, Germany) so the hip, knee and ankle were all fixed. After fracture and casting, the mice were given subcutaneously 2 days of buprenorphine (0.1 mg/kg) and enrofloxacin (5 mg/kg) as well as 1.0 ml of normal saline. At 3 weeks after surgery the mice were anesthetized with isoflurane and the cast removed. All mice had union at the fracture site by manual inspection.

Hindpaw nociceptive testing

Mechanical allodynia was assayed using nylon von Frey filaments according to the "up-down" algorithm as previously described [33]. The mice were placed on wire mesh platforms in clear cylindrical plastic enclosures 10 cm in diameter and 40 cm in height, and after 15 minutes of acclimation von Frey fibers of sequentially increasing stiffness were applied against the hindpaw plantar skin at approximately midsole, taking care to avoid the tori pads, and pressed upward to cause a slight bend in the fiber and left in place for 5 sec. Withdrawal of or licking the hindpaw after fiber application was scored as a response. When no response was obtained the next stiffest fiber in the series was applied to the same paw; if a response was obtained a less stiff fiber was applied. Testing proceeded in this manner until 4 fibers had been applied after "negative+positive or positive+negative" response. Hindpaw testing was performed bilaterally. Estimation of the mechanical withdrawal threshold by data fitting algorithm permitted the use of parametric statistics for analysis [34]. These data were analyzed as the difference between the fracture side and the contralateral untreated side, thus a negative value represents a reduction in the fracture hindpaw withdrawal threshold.

An incapacitance device (IITC Life Science, Woodland Hills, CA) was used to measure bilateral hindpaw weight bearing. The mice were manually held in a vertical position over the apparatus with the hindpaws resting on separate metal scale plates and the entire weight of the mouse was supported on the hindpaws. The duration of each measurement was 6 s and 6 consecutive measurements were taken at 10 s intervals. All 6 readings were averaged to calculate the bilateral hindpaw weight-bearing values. Right hindpaw (fracture side) weight-bearing data were analyzed as a ratio between the right hindpaw weight and the average of right

and left hindpaw values ((2R/(R + L)) × 100%), thus a value less than 100% represents a decrease in weight bearing in the fracture limb.

CRPS IgM immunoglobulin purification from CRPS sera

After informed consent was obtained and the clinical evaluation was completed, CRPS patient and control subject blood was collected, centrifuged, and the serum supernatants were aliquoted and frozen at - 80°C. IgM was extracted from chronic CRPS patient and control subject serum using a polypropylene column (BioRad, Hercules, CA) pre-packed with POROS CaptureSelect™ IgM Affinity Matrix (Thermo fisher Scientific, Leiden, Netherlands). The bound IgM was eluted using 100mM glycine pH 3, then the elute pH was adjusted to 7.4 using 1M Tris pH 8.0, then Slide-A-Lyzer Dialysis Cassettes (3.5K MWCO, Life Technologies, Carlsbad, CA) were used to remove glycine from protein and the IgM quantified using Bio-Rad Protein Assay (Hercules, CA). The dose of IgM used in the current study (1 ug) is 0.2% the amount of IgM extracted from 500 ul of human serum.

Dot blot assays for screening CRPS IgM binding reactivity to glial fibrillary acidic protein

Recombinant human glial fibrillary acidic protein (GFAP), a protein primarily found in astrocytes, was purchased from Abcam (Burlingame, CA) for dot blotting to measure specific IgM binding reactivity in the sera of normal controls, early CRPS patients, chronic CRPS patients, and resolved CRPS patients. This autoantigen was selected based on: 1) the results of our previous studies demonstrating that intrathecal injections of both early and chronic CRPS patient IgM have pronociceptive effects in muMT mice [4,8], and 2) our previous liquid chromatography-tandem mass spectroscopy studies in the skin and spinal cord of fracture mice suggesting that early CRPS patient IgM had increased GFAP binding affinity[9]. The GFAP recombinant protein (2ul) was applied to a nitrocellulose membrane and incubated for 1 hour with human sera (1:1000 dilution in TPBS) followed by 1hour incubation with anti-human IgM-Dylight800 secondary antibody (1:10000 dilution, Thermo Fisher Scientific, Waltham, MA). The signals were detected using Odyssey Near-Infrared Fluorescence Imaging System and quantified using Image Studio Lite (LI-COR Biosciences, Lincoln, NE).

Quantitative real-time polymerase chain reaction assays for IgM induced inflammatory cytokine expression in the spinal cord of fracture mice

We postulated that the pronociceptive effects of chronic CRPS IgM intrathecal injection were mediated by spinal C5a complement signaling in microglia evoking inflammatory cytokine expression, thus resembling the pronociceptive mechanisms observed with early CRPS IgM intrathecal and intraplantar injections [8]. After baseline nociceptive behavioral testing, muMT mice underwent tibia fracture and casting. At 3 weeks post fracture, the casts were removed and the next day nociceptive behavioral testing was repeated and the mice were then intrathecally injected with either pooled chronic CRPS patient IgM (1 ug/5ul, i.t.) or pooled control subject IgM, either with or without the co-injection of the C5a receptor antagonist PMX-53 (200ng/5ul, R&D Systems, Minneapolis, MN). At 1 hour after IgM/PMX-53 injection the muMT mice were euthanized and the L4,5 lumber spinal cord tissues were collected. Total RNA was extracted by using the RNeasy Mini Kit (Qiagen, Redwood City, CA), and the purity and concentration were determined spectrophotometrically. Then complementary DNA was synthesized from 1 µg RNA using RT² First Strand Kit (Qiagen). Real-time polymerase chain reactions were performed on the QuantStudio 7 Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA) using RT² qPCR Primer Assay and the RT² SYBR Green qPCR Mastermix (Qiagen, Redwood City, CA). Tumor necrosis factor alpha (TNF), interleukin 1 beta (IL-1), interleukin 6 (IL-6), and 18S primer sets were validated on dissociation curves to document single product formation. The data from real-time polymerase chain reaction experiments were analyzed by the comparative CT ($2^{-\Delta\Delta Ct}$) method as described in the manufacturer's manual. All results were confirmed by repeating each experiment twice. Data collection was conducted blind to treatment assignment.

Enzyme immunoassay for evaluating TNF and IL-1 protein levels in the lumbar spinal cords of fracture muMT mice

After baseline nociceptive behavioral testing, muMT mice underwent right tibia fracture and casting. At 3 weeks post fracture, the casts were removed and the next day nociceptive behavioral testing was repeated and the mice were then intrathecally injected with either pooled chronic CRPS patient IgM (1 ug/5ul, i.t.) or pooled control subject IgM. At 1 hour after IgM injection the muMT mice were euthanized and the right side L4,5 lumber spinal cord tissues were collected and frozen immediately on dry ice. The spinal cord tissue was cut into fine pieces

in ice-cold PBS, pH 7.4, containing a cocktail of protease inhibitors (Roche Applied Science) followed by homogenization using TissueLyser LT (QIAGEN). Homogenates were centrifuged for 15min at 12,000g, 4°C. The supernatants were aliquoted and stored at -80°C until required for enzyme immunoassay (EIA) performance. Total protein contents in all tissue extracts were measured using the DC protein assay kit (Bio-Rad). TNF and IL-1 levels were determined in duplicate using respective EIA kits (R&D Systems), according to the manufacturer's instructions. These assay systems detect mouse TNF and IL-1 with sensitivity of 0.148pg/ml and 2.31pg/ml, respectively. The concentrations of both proteins were calculated from the standard curve at each assay. Each protein concentration was expressed as pg/mg total protein.

Chronic CRPS patient IgM injection experiments in muMT fracture mice

Intrathecal and intradermal injection experiments with individual chronic CRPS IgM samples

These experiments examined the pronociceptive (hindpaw mechanical allodynia and unweighting) effects of chronic (>12 month post injury) CRPS patient IgM after intrathecal or intraplantar injection into 3 weeks post fracture muMT mice. The muMT mice underwent baseline behavioral testing (measuring bilateral hindpaw von Frey withdrawal thresholds and hindpaw weight bearing) and then, under isoflurane anesthesia, the right distal tibia was fractured and casted. At 3 weeks post fracture the casts were removed and behavioral testing was repeated the next day and then chronic CRPS patient IgM or with normal control subject IgM was injected into the L5/L6 intrathecal space (1ug/5ul, IT.) or injected intradermally (1ug/10ul, IPL) into the plantar skin of the fracture limb hindpaw. Further behavioral testing was performed at 1 hour after injection (time of peak pronociceptive effect for early CRPS patient IgM) [4]. Each CRPS patient (n=10) or control (n=10) serum was injected into 3 different muMT fracture mice and the test results were averaged for each subject. Data collection was conducted blind to treatment assignment.

Pronociceptive effects of intrathecal and intradermal anti-GFAP antibody experiments

Because early and chronic CRPS patient IgM exhibit increased binding affinity to the glial fibrillary acidic protein (GFAP) [9], we postulated that the intrathecal injection of an anti-GFAP antibody into muMT fracture mice would be pronociceptive, but the intradermal injection of this anti-GFAP antibody would have no effect in muMT fracture mice. The pronociceptive effects of

intrathecal and intradermal intraplantar injection of a commercially available mouse polyclonal IgG antibody (SAB1405864, Sigma-Aldrich, Saint Louis, MO) targeting a broad range of human GFAP epitopes were evaluated in 3-week post fracture muMT mice. Sigma normal mouse IgG (I8765) was used as the Control IgG for the anti-GFAP antibody per Sigma's recommendation. The gene GFAP encodes an intermediate filament protein (47.5 kDa) in mature astrocytes. MuMT mice underwent baseline nociceptive behavioral testing and tibia fracture and casting, then at 3 weeks post fracture the casts were removed. The next day nociceptive behavioral testing was repeated and then anti-GFAP IgG antibody or control IgG was injected intrathecally (1ug/5ul PBS) or intraplantarly (1ug/10ul) and further nociceptive behavioral testing was performed at 0.5, 1, 3, 6, and 24 hours post injection. Another cohort of nonfracture muMT mice underwent right hindpaw von Frey withdrawal threshold testing and then were administered either; 1) intrathecal anti-GFAP IgG (1ug/5ul PBS), 2)GFAP (0.1ug/5ul PBS, OPCD00706, Avia Systems Biology, San Diego, CA), or 3) an immune complex of anti-GFAP IgG (1ug) bound to GFAP (0.1ug) and then hindpaw von Frey testing was repeated at 0.5, 1, 3, 6, and 24 hours post injection. The anti-GFAP IgG and GFAP solution was premixed and then incubated at 4° C overnight to form anti-GFAP IgG-GFAP immune complexes for intrathecal injection. Data collection was conducted blind to treatment assignment.

Effects of mannitol disruption of the blood-spinal cord barrier and pronociceptive onset latency

Another experiment examined the hypothesis that the striking differences in pronociceptive onset latencies after systemic (7 days) and localized (1 hour) injections of fracture mouse and CRPS patient IgM into muMT fracture mice were due to restricted access of circulating IgM to the hindpaw epidermal space and its corresponding innervation in the lumbar spinal cord. The epidermis normally lacks vascular supply and the spinal cord is normally isolated from the adaptive immune system by the blood-spinal cord barrier. Mannitol, a well-characterized osmotic agent, when clinically administered by intravascular infusion induces transient blood brain barrier permeability to IgM [35] and a wide array of various other large proteins, with peak effect developing within 5 minutes and persisting for 20-30 minutes [36].

After baseline nociceptive behavioral testing, muMT mice underwent tibia fracture and casting. At 3 weeks post fracture the casts were removed and the following day nociceptive behavioral testing was repeated and then the mice were intraperitoneally injected with 3-week

wildtype fracture mouse serum (0.5ml, i.p.). Two days after serum injection the behavioral testing was repeated and then the mice underwent a retro-orbital intravenous mannitol injection (2mg/g body weight in 20% solution, 2uL/sec, ICU Medical Inc, Lake Forest, IL) using a 30-gauge 8mm long needle. Further nociceptive behavioral testing was performed at 0.5, 1, 3, 6, and 24 hours and 7, 9, 14, and 21 days after intravenous injection. An additional group of muMT fracture mice underwent the same protocol but were injected with saline instead of mannitol. A similar experiment was performed using intraperitoneally injection of chronic CRPS patient IgM (0.5 mg/0.5ml saline, i.p.) in 3-week muMT fracture mice and 2 days later injecting intravenous mannitol or saline. Data collection was conducted blind to treatment assignment.

Pronociceptive IgM and the role of C5a complement signaling experiments

To test the hypothesis that the pronociceptive effects of chronic CRPS IgM were mediated by C5a complement signaling, thus resembling the pronociceptive mechanisms observed with early CRPS IgM intrathecal and intraplantar injections [8], muMT mice underwent baseline nociceptive behavioral testing and then tibia fracture and casting. At 3 weeks post fracture the casts were removed and the next day nociceptive behavioral testing was repeated and the mice were then intrathecally injected with either pooled chronic CRPS patient IgM (1 ug/5ul, i.t.) or pooled control subject IgM, either with or without the co-injection of the C5a receptor antagonist PMX53 (200ng/5ul, R&D Systems, Minneapolis, MN). Further nociceptive behavioral testing was performed at 0.5, 1, 3, 6, and 24 hours after injection. This same experiment was repeated without nociceptive behavioral testing and at 1 hour after IgM IT injection the muMT mouse spinal cords were collected for TNF-α, IL-1β, and IL-6 real-time polymerase chain reaction assays. Data collection was conducted blind to treatment assignment.

Pronociceptive IgM and the role of inflammatory cytokines experiments

An additional experiment tested the hypothesis that chronic CRPS IgM pronociceptive effects required cytokine signaling in the spinal cord, similar to the cytokine dependent pronociceptive effects of early CRPS IgM after intrathecal injection.[8] MuMT mice underwent baseline nociceptive behavioral testing and then tibia fracture and casting. At 3 weeks post fracture the casts were removed and the next day nociceptive behavioral testing was repeated and then the global cytokine inhibitor pentoxifylline (Sigma Aldrich, St. Louis, MO) or plain water

was administered by gavage for 2 consecutive days (200mg/kg, daily), then the mice were intrathecally injected with pooled chronic CRPS patient IgM (1 ug/5ul, i.t.). Further nociceptive behavioral testing was performed at 0.5, 1, 3, 6, and 24 hours post injection. Another cohort of muMT fracture mice were administered pentoxifylline but were not injected with IgM. Data collection was conducted blind to treatment assignment.

Statistical analysis

Statistical analysis was performed using an unpaired t-test (Fig. 1), a two-way repeated measures ANOVA (Figs. 3,4,5,8) or a one-way ANOVA (Figs. 2,6) with Holm-Sidak multiple comparisons test for post-hoc contrasts. Data are presented as the mean ± standard error of the mean or scatter plots, and differences are considered significant at a P value less than 0.05 (Prism 10, GraphPad Software, San Diego, CA). Mouse cohort sizes were based on a power analysis of our preliminary and previously published data generated from using each of the proposed assays (allodynia, unweighting, cytokine PCR measurements, and antigen binding affinity) in fracture animals. Based on this analysis we calculated that the proposed experiments require 6 to10 mice per cohort to provide 80% power to detect 25% differences between groups. No mice were excluded from the statistical analyses of experimental results.

Declaration of competing interest

The authors do not have financial or other relationships that might lead to conflict of interest.

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Data availability

The data analysis in this study was performed using Prism 10 software (GraphPad Software, San Diego, CA) and these Prism files are available to any researcher requesting them from the corresponding author.

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