**Cartilage binding antibodies induce pain through immune complex mediated stimulation of neurons**

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**Summary (40 words)**

This work elucidates a novel pain mechanism generated by rheumatoid arthritis-associated autoantibodies, uncoupled from inflammation, which is dependent on immune complex formation and activation of neuronal FcγRI.

**Abstract**

Rheumatoid arthritis-associated joint pain is frequently observed independent of disease activity, suggesting unidentified pain mechanisms. We demonstrate that antibodies binding to cartilage, specific for collagen type II (CII) or cartilage oligomeric matrix protein (COMP), elicit mechanical hypersensitivity in mice, uncoupled from visual, histological and molecular indications of inflammation. Cartilage antibody-induced pain-like behavior does not depend on complement activation or joint inflammation, but instead on tissue antigen recognition and local immune complex (IC) formation. smFISH and IHC suggest that neuronal *Fcgr1 and Fcgr2b* mRNA are transported to peripheral ends of primary afferents. CII-IC directly activate cultured WT but not FcRγ-chain deficient DRG neurons. In line with this observation, CII-IC does not induce mechanical hypersensitivity in FcRγ-chain deficient. Furthermore, injection of CII antibodies does not generate pain-like behavior in FcRγ-chain deficient mice or mice lacking activating FcγRs in neurons. In summary, this study defines functional coupling between autoantibodies and pain transmission that may facilitate the development of new disease-relevant pain therapeutics.

**Keywords**

Collagen type II autoantibodies, antibody-induced arthritis, immune complex, sensory neurons, pain, Fcγ receptors, rheumatoid arthritis

**Introduction**

The molecular dialog between the immune system and nociceptive neurons is a fundamental aspect of both acute and chronic pain. In particular the contribution of the adaptive immune system has recently come into focus. Reports show that autoantibodies against specific neuronal proteins increase the excitability of nociceptors without involvement of other inflammatory factors (Dawes et al., 2018; Klein et al., 2012). For instance, autoantibodies against components of the voltage-gated potassium channel complex isolated from patients with Morvan’s disease can directly elicit hyperexcitability in specific subsets of nociceptive neurons and cause neuropathic pain (Dawes et al., 2018; Klein et al., 2012). Similarly, autoantibodies have been suggested to cause pain in rheumatoid arthritis (RA). Recent studies demonstrate that individuals can be seropositive for RA-associated autoantibodies such as rheumatoid factor and anti-citrullinated protein antibodies (ACPA) for several years prior to clinical onset of the disease (Rantapaa-Dahlqvist et al., 2003) and antibodies present during early stages of arthritis can interact with joint cartilage and collagen type II (CII) (Haag et al., 2014; Pereira et al., 1985). During the period immediately prior to diagnosis individuals frequently suffer from joint pain, often without signs of joint inflammation (de Hair et al., 2014). We have previously reported that antibodies reactive with cartilage CII can cause mechanical hypersensitivity without visible joint inflammation when injected into mice (Bas et al., 2012). Furthermore, pain still persists in a sizable proportion of RA patients for whom other RA symptoms, including joint inflammation, are medically controlled (Taylor et al., 2010). Thus, joint pain uncoupled from apparent disease activity is a pervasive problem and represents a fundamental gap in our mechanistic understanding of pain in autoimmune disorders.

A subgroup of RA patients displays elevated levels of circulating and intra-synovial anti-CII antibodies around the time of RA diagnosis, though their precise frequency is debated (Clague and Moore, 1984; Pereira et al., 1985). CII is a structural protein mainly found in articular cartilage, and rodents and primates immunized with CII develop an autoimmune response and joint pathology similar to human RA (Lindh et al., 2014). The transfer of monoclonal anti-CII antibodies to rodents causes a similar pathological state (Holmdahl et al., 1986; Terato et al., 1992), which is the basis for the collagen antibody-induced arthritis (CAIA) model (Nandakumar et al., 2003). When we assessed pain-like behavior in the CAIA model we found that mechanical hypersensitivity develops prior to any signs of joint inflammation and remains for weeks after inflammation has subsided (Bas et al., 2012; Su et al., 2015). Anti-CII antibodies cause denaturation of collagen fibrils and loss of chondrocytes *in vitro* (Amirahmadi et al., 2005) and early loss of proteoglycans *in vivo,* without the influence of inflammation (Nandakumar et al., 2008). However, as cartilage is not innervated the anti-CII antibodies must act on other targets to mediate pro-nociceptive effects in the pre-inflammatory stage. Thus, the aim of this study was to investigate the pro-nociceptive properties of anti-CII antibodies.

**Results**

**Induction of pain-like behavior by anti-CII antibodies is not associated with inflammation**

CAIA was induced by injection of an anti-CII mAb cocktail followed by LPS 5 days later. Cell infiltration, bone erosion and cartilage destruction were readily detectable by day 15. We observed that mice not only displayed a reduction in tactile thresholds during the disease phase, but that mechanical hypersensitivity was already present prior to visible joint inflammation, on days 3 and 5 (Fig. 1A-C). Although no ankle-joint pathology was observed prior to day 5, synovitis was present in 2 out of 8 with coincident arthritis scores of 5 and 13 on a scale of 1-60 (Fig. 1D-G). No correlation was found between **Von Frey pain-like behavior** and arthritis scores at day 5 (r=0.159, p=0.634, n=19).

In order to determine if anti-CII mAbs, in the absence of LPS, induce a low-grade inflammation capable of activating sensory neurons, joints were processed for molecular analysis of factors associated with arthritis pathology and pain signaling. While mRNA levels of tumor necrosis factor (*Tnf*), interleukins *Il-1b* and *Il-6*, prostaglandin-producing enzyme cyclooxygenase-2 (*Cox2*), mast cell proteases (*Mcpt4*), and matrix metalloproteases *Mmp2*, *Mmp9* and *Mmp13* were significantly increased at day 15 of the CAIA model, none of them were elevated 5 days after injection of the anti-CII mAb cocktail when compared to saline controls (Fig. 1H-I). Furthermore, changes in MMP activity were examined using MMPsense. An increase in fluorescent signal was detected in the paws 15 days after the injection of anti-CII mAb cocktail, but again no differences were observed between antibody-injected mice and saline controls on day 5, suggesting that MMPs were not activated at this time point (Fig. 1J-K). Taken together, these results suggest that factors other than innate inflammatory and extracellular matrix remodeling mediators drive anti-CII mAb induced mechanical hypersensitivity prior to onset of joint inflammation. Thus, in the subsequent studies we focused on the early phase of the CAIA model (days 0-5; prior to LPS injection) in order to explore the mechanisms by which anti-CII mAbs induce pain-like behavior prior to inflammation.

**Pain-like behavior is apparent as early as two days after injection of anti-CII antibodies**

When assessed daily, we found that the anti-CII mAb cocktail induced a significant reduction in tactile thresholds by day 2 compared to saline injected animals (Fig. 2A). None of the mice displayed signs of joint inflammation prior to day 4, and only 3 out of 14 developed mild signs of joint inflammation by day 5, characterized by arthritis scores ranging from 5-13 (Fig. 2B-C). Reduction in locomotor activity has been used as a surrogate of pain-related mobility impairment in rodents (Cho et al., 2013). When assessed during the third night (12-hour period) after antibody injection, a reduction in total movement and rearing activity was detected (Fig. 2D), suggesting that anti-CII mAbs decrease voluntary and spontaneous locomotion in mice prior to signs of inflammation. In contrast, when we performed the inverted grid test, none of the mice injected with anti-CII mAbs (day 5) or saline (n=10/group) fell from the fully turned grid, indicating that both groups displayed similar grip and muscular strength.

**Antibody epitope recognition, but not pathogenicity is important in early anti-CII antibody induced pain-like behavior**

In order to investigate whether the arthritogenic potency of different CII mAbs correlates to their pronociceptive potency, we injected the antibodies individually and measured mechanical sensitivity. All four antibodies, but not the isotype control antibodies, induced similar degrees of mechanical hypersensitivity when injected individually (Fig. 2E-G), as well as in combination (Fig. 2A). Injection of the anti-CII mAb M2139 alone, the most arthritogenic antibody in the cocktail, also reduced total movement compared to the isotype IgG2b control antibody, although the difference in rearing between the groups did not reach statistical significance (p=0.054) (Fig. 2H). M2139 induced mechanical hypersensitivity at different doses (Fig. 2I), which lasted up to 21 days following a single injection, even with doses of M2139 that failed to induce joint inflammation at any time point (Fig. 2J-K).

**Complement factor C5 and changes in cartilage structure do not contribute to early pain-like behavior**

To examine if anti-CII mAbs induce nociception through activation of the complement cascade, PMX53, a cyclic peptide C5aR antagonist, was injected daily starting one day prior to administration of the anti-CII mAb cocktail (day 0). The C5aR antagonist failed to reverse antibody-induced changes in mechanical hypersensitivity or locomotor activity (Fig. 3A-C). Furthermore, the degree of mechanical hypersensitivity and reduction in locomotion were not different between B10Q.C5\* mice lacking functional C5 and WT mice subsequent to injection of anti-CII mAbs (Fig. 3D-F). As previously shown, the CIIF4 antibody binds to CII but does not lead to cartilage damage (Burkhardt et al., 2002; Croxford et al., 2010; Nandakumar et al., 2008). Injection of CIIF4 antibody also induced robust mechanical hypersensitivity comparable to the other anti-CII mAbs tested (Fig. 3G). These experiments indicate that the mechanism responsible for anti-CII antibody-mediated nociception is independent of C5 (and thereby terminal/lytic complement) or changes in cartilage structure.

**FcγRs are present in mouse sensory neurons**

As an alternative pronociceptive mechanism, we explored interactions between CII-ICs and neurons. We examined expression of FcγRs in mouse sensory neurons using several different techniques. First, we observed the mRNA expression of all four *Fcgr*  (*Fcgr1, Fcgr2b, Fcgr3*, and *Fcgr4)* in mouse DRG via gene expression microarrays (Fig. 4A), which were subsequently confirmed by quantitative real time PCR (Fig. 4B). These data are in line with the publicly available resource DRG XTome database (Ted Price laboratory, University of Texas at Dallas, Dallas, USA), which shows the presence of *Fcgr* mRNA in mouse DRGs (Fig. 4C) (Ray et al., 2018). Using smFISH, we detected mRNA molecules for *Fcgr1, Fcgr2b*and*Fcgr3* in both neuronal (co-localizing with NeuN) and non-neuronal cells, but failed to detect *Fcgr4* (Fig. 4D)*.*Quantification of single mRNA molecules for each receptor in individual sensory neurons plotted by area of neuronal soma, showed the highest expression of *Fcgr1* in neurons (Fig. 4D and S1). Using WB for protein analysis, FcγRI was detected in DRG and spleen (positive control) homogenates from WT but not from FcRγ-chain-/-mice, which lack cell surface expression and signaling of all activating FcγRs (I, III and IV) (Fig. 4E) (Takai et al., 1994). As FcγRIIb and FcγRIII antibodies did not work for WB, we examined their presence in full DRGs lysates with high performance nanoLC-MS/MS proteomics, revealing the presence of FcγRIIb based on the identification of two unique peptides originating from FcγRIIb (Fig. 4F), along with two peptides that are shared between FcγRIIb and FcγRIII (data not shown).

Cellular localization was examined via IHC. FcγRI immunoreactivity was detected in DRGs of WT BALB/c and C57BL/6 mice (Fig. 5A and S2), co-localizing with Iba1-positive resident macrophages (Fig. 5B), but not satellite cells or neurons (lack of co-localization with vimentin and TrkA, respectively) (Fig. S2). As FcγRI is expressed in the soma of rat DRG neurons (Jiang et al., 2017; Qu et al., 2012; Qu et al., 2011) we verified our finding by employing anti-FcγRI antibodies from several vendors (Fig. S2 and S3) and using DRG sections from FcRγ-chain-/-mice as a negative control. Three out of four antibodies tested on DRG sections labeled resident macrophages in WT mice with no detectible signal in FcRγ-chain-/- mice, and the fourth displayed non-specific labeling in all sections (Fig. 5A and S3).

In contrast, FcγRIIb protein expression was detected in the soma of DRG neurons, confirmed by co-localization with neuronal marker TrkA (Fig. 5C-D). As expected, FcγRIIb signal was still present in FcRγ-chain-/-mice (Fig. 5C). No immunoreactivity was detected for FcγRIII and FcγRIV (Fig. S2). Both FcγRI and FcγRIIb immunoreactivity were detected in glabrous skin sections, and although FcγRI was expressed exclusively in macrophages in the DRG, both FcγRI and FcγRIIb co-localized with neuronal marker PGP9.5 in the skin (Fig. 5E-F). Both receptors were also detected in non-neuronal cells in the skin.

Due to the lack of FcγRI protein expression in the cell bodies of DRG neurons, the presence of FcγRI immunoreactivity in the end structures of PGP9.5 positive neurons and the high levels of *Fcgr1* mRNA in non-neuronal, non-nuclear areas in the DRG that contain mainly fiber tracts (Fig. S4) we examined whether *Fcgr1* mRNA may be transported down the axon for local translation. smFISH performed after ligation of the sciatic nerve (Fig. 5G) showed that *Fcgr1* and *Fcgr2b* mRNA molecules accumulated proximal to the ligature within fiber tracts, as compared to the contralateral nerve, indicating axonally transported mRNA (Fig. 5H-I). In conclusion, both FcγRI and FcγRIIb are expressed in sensory neurons, however, while FcγRIIb protein was detected both in the DRG cell body and axons, FcγRI protein was only detected in the peripheral axon *in vivo*.

**CII-IC activates cultured DRG neurons causing increased intracellular [Ca2+]** **and inward current**

Primary DRG neuronal cell cultures were used for *in vitro* experiments. FcγRI and FcγRIIb immunoreactivity co-localized with the neuronal marker (βIII-tubulin); FcγRIIb expression was more pronounced in the cell bodies, and FcγRI immunoreactivity was more prominent in the axons and neurites (Fig. 6A). Immunoreactivity for FcγRIII and FcγRIV were not detected (data not shown). Primary DRG neurons stimulated with CII-IC exhibited an increased intracellular [Ca2+] signal in 247 cells (22.1%) out of 1119 viable neurons (KCl responding), while stimulation with monomeric control IgG2b evoked response in less than 1% of viable neuronal cells (Fig. 6B).

We then performed electrophysiological recordings on a subpopulation of nociceptive neurons that express transient receptor potential vanilloid 1 (TRPV1) receptors. Capsaicin (0.5 µM), a potent TRPV1 agonist, was added at the end of each experiment to verify neuronal population and viability. In total, 114 cells were patched and ionic currents were recorded in whole-cell voltage clamp mode. Of the 114 cells, 52 cells showed an inward current in response to capsaicin (48%). Stimulation of capsaicin responding neurons with CII-IC evoked inward currents in 42% (22 out of 52, 19% of total cells), while stimulation with IgG2b failed to evoke inward currents in any of the 18 neurons assayed (Fig. 6C). Moreover, we performed a similar experiment applying a generic IC (mouse anti-rat IgGs and as antigen rat-IgGs) and found that 10 out of 46 total cells (22%) patched showed inward currents in response to IgG-IC.

**CII-IC induces CGRP release in primary DRG cultures from WT but not FcγR-chain-/- mice**

CGRP is a neuropeptide, expressed in nociceptive neurons, released upon various noxious stimuli (van Rossum et al., 1997). A bell-shaped dose-response relationship was observed upon DRG stimulation with 0.1-10 µg/ml of CII-IC; with the largest CGRP release induced by 1 µg/ml of CII-IC (Fig. 6D). In contrast, CGRP levels in the DRG culture supernatants were not elevated in response to stimulation with monomeric anti-CII mAbs, CII or control IgG2b antibody (Fig. 6D). Furthermore, while primary DRG neuronal cultures established from FcRγ-chain-/- mice responded to stimulation with the positive control, capsaicin, stimulation with CII-IC did not induce CGRP release (Fig. 6E). Finally, in Ca2+ imaging experiments there was no difference in percentage of neurons responding to CII-IC in cultures established from FcγRIII-/- (10/85 viable cells) and WT (8/92 viable neurons) mice (respectively 8.7% and 11.8%). These results suggest that the neuronal high affinity FcγRI, rather than the low affinity FcγRIIb, is responsible for CII-IC-induced release of CGRP *in vitro*.

**Intra-articular injection of IC induces pain-like behavior**

To investigate whether CII-IC induces pain-like behavior *in vivo*, we injected CII-IC into the intra-articular (i.a.) space of the ankle joint. I.a. injections of both CII-IC (Fig. 7A), as well as a general immune complex (Fig. 7D) elicited mechanical hypersensitivity in the ipsilateral paw at 1 or 3 hours after injection. Furthermore, i.v. injection of an antibody to COMP (Geng et al., 2012), a major non-collagenous component of cartilage, and i.a. injection of COMP-IC, induced pain-like behavior in the absence of inflammation (Fig 7E-G). In contrast, neither i.a. nor i.v. injection of CII-IC or anti-CII mAbs induced mechanical hypersensitivity in FcRγ-chain-/-mice (Fig. 7B-D). Finally, systemic injection of anti-CII mAbs induced mechanical hypersensitivity in FcγRIV-/- mice, which persisted at 30 days despite these mice being resistant to induction of CAIA (Fig. 7H-J). These data suggest that activating FcγRs, and FcγRI in particular, are critical for development IC-mediated pain-like behavior *in vivo*.

**Intact and glycosylated CII antibodies are required for pro-nociceptive effect *in vivo***

Fab fragments of the four anti-CII mAbs did not induce signs of mechanical hypersensitivity or altered locomotion upon i.v. injection (Fig. 8A-C), indicating the Fc-portion as necessary for development of pain-like behavior. Moreover, EndoS-treated anti-CII mAb M2139 injected i.v. did not induce mechanical hypersensitivity or reduced locomotion compared to control mice, even though a robust change in behavior was observed in mice receiving intact M2139 anti-CII mAbs (Fig. 8E-G). Similarly, EndoS-treated anti-CII mAb cocktail did not reduce withdrawal thresholds (Fig 8D). These observations taken together indicate that the binding of Fab to CII alone, is not capable of activating nociceptors and that glycosylation of Fc is required.

**FcγRs on neurons and not hematopoietic cells are responsible for anti-CII antibody induced pain-like behavior**

We used chimeric mice to investigate the contribution of FcγRI to anti-CII antibody-induced pain in hematopoietic cells as compared to non-hematopoietic cells (including neurons). Mice were irradiated to deplete hematopoietic cells and then transplanted with bone marrow from either WT or FcRγ-chain-/- mice. Irradiated WT mice that received WT bone marrow were used as a control. Three groups of mice (WT-KO, KO-WT and WT-WT) were injected with either saline or anti-CII mAbs and monitored for mechanical thresholds for 6 days. Mice expressing activating FcγRs solely on hematopoietic cells and not neurons or other non-hematopoietic cells (WT-KO) were protected from anti-CII antibody-induced mechanical hypersensitivity, while mice lacking FcγRs on myeloid cells (KO-WT) developed mechanical hypersensitivity indistinguishable from control mice (WT-WT, Fig. 8H-J). While these results do not exclusively test the role of activating FcγRs on neurons, they indeed support such a link, as FcγRs on immune cells are not critical for induction of anti-CII mAb-mediated pain-like behavior.

**Activating FcγRs are expressed by human sensory neurons**

Data from the publicly available DRG XTome database (Ted Price laboratory, University of Texas at Dallas, Dallas, USA) show the presence of *Fcgr* mRNA in human DRGs, with *Fcgr3A* being the highest expressed (Figure 9A) (Ray et al., 2018). Using IHC, we examined protein expression of activating FcγRs in human DRGs (n=4) and found that FcγRI expression did not co-localize with the neuronal marker NeuN but was present in cells with a macrophage-like morphology (Fig. 9B-C). Using antibodies against FcγRIIa, FcγRIIIb and FcγRIIIa/b we were only able to detect a positive signal from FcγRIIIa/b, which colocalized with NeuN positive neurons as well as non-neuronal macrophage-like cells in the human DRGs (Fig. 9D-E).

**Discussion**

In the present study, we have explored the mechanisms by which anti-CII antibodies induce pain-like behavior prior to induction of arthritis. We provide evidence that these antibodies trigger pain-like behavior prior to any visual, histological or molecular signs of inflammation, independently of complement factor C5 and changes in cartilage integrity. By using modified anti-CII antibodies and transgenic or chimeric mice we established that, in addition to epitope recognition, interaction with neuronal FcγRI is critical for the pro-nociceptive properties of anti-CII antibodies. Lastly, the presence of FcγRIII in human DRG neurons suggests the translation potential of this work. While further studies are warranted, our findings support a role for neuronal FcγRs in autoimmune pain conditions.

Collagen type II antibodies readily bind joint cartilage *in vivo*, forming ICs which are likely crucial for the attraction of inflammatory cells and represent a key step in arthritis development. Thus, we initially hypothesized that the early pain-like behavior following injection of anti-CII antibodies is mediated by local soluble CII-IC, inducing a low grade, local inflammation, not detectable as swelling. However, we did not find any indications of coupling between inflammatory processes and nociception during the first five days after antibody injection in the joints. Even though activation of inflammatory cells is often indicated as necessary for induction of RA pathogenesis, arthritogenic antibodies can directly cause cartilage destabilization both *in vitro* and *in vivo*, preceding the onset of arthritis, independently of inflammation (Nandakumar et al., 2008). We asked if associated actions could also drive pro-nociceptive processes. To test this, we used CIIF4, an antibody that unlike the other CII mAbs used in this study, binds CII but lacks arthritogenicity. In fact this antibody is protective when given together with other anti-CII antibodies both *in vivo* and *in vitro* (Croxford et al., 2010; Nandakumar et al., 2008). Remarkably, CIIF4 induced pronounced mechanical hypersensitivity. Thus, the processes associated with anti-CII antibody-induced cartilage loss unlikely mediate pain-like behavior induced by the antibodies. Together these experiments led us to conclude that neither inflammation, terminal/lytic complement nor cartilage breakdown are mechanistic explanations for the pain-like behavior observed in the early phase of CAIA. Prompted to explore alternative mechanisms we turned our attention to direct actions of anti-CII antibodies on peripheral neurons.

In order to examine if anti-CII antibodies have a direct action on nociceptors we added the antibodies in monomeric form to DRG neurons in culture. While the cells responded to positive controls, no increase in inward currents, intracellular [Ca2+] or release of the pain-associated neuropeptide CGRP were observed in the presence of anti-CII or control antibodies. This is not surprising as CII displays a very narrow tissue distribution. Except for hyaline cartilage in joints, it is found in the ear, larynx, trachea, vitreous of the eye (Eyre, 1991) and in thymus (Raposo et al., 2018). We also did not detect any anti-CII positivity via Western blots from DRG lysates or primary DRG cultures (data not shown). Hence, we concluded that there are no CII epitopes present on the neuronal membrane and that a direct neuronal action of monomeric anti-CII antibodies via the Fab region is unlikely. However, the Fc region of IgG antibodies in the context of ICs can activate FcγRs. Intriguingly, several studies show expression of FcγRI in rat sensory neurons (Andoh and Kuraishi, 2004; Qu et al., 2012; Qu et al., 2011) and here we report that in addition to FcγRI, mouse DRG neurons also express FcγRIIb *in vivo* and *in vitro*. Anti-CII antibodies in IC with CII evoked inward currents and increased intracellular [Ca2+] along with release of the pain-associated neuropeptide CGRP in mouse cultured primary DRG neurons. Thus, the presence of immune cells was not necessary for IC mediated activation of nociceptive neurons, which supports the notion of a direct link between antibodies and regulation of neuronal excitability. Our results are consistent with previous findings showing that IgG-ICs increase intracellular Ca2+ levels, membrane depolarization and release of substance P from cultured DRG neurons (Andoh and Kuraishi, 2004; Jiang et al., 2017; Qu et al., 2011). Neuronally expressed FcγRI has been coupled to the activation of the cation channel TRPC3 through a signaling pathway involving Syk, PLC, and the IP3 receptor (Qu et al., 2012). In previous studies examining the effect of IC activation of neuronal FcγRs, exogenous antigens (normal mouse IgG or [ovalbumin](https://www.sciencedirect.com/topics/neuroscience/ovalbumin), OVA) have been utilized to generate immune complexes with rat anti-mouse IgG or anti-OVA IgG, respectively. In the current study we employed a model, which mimics the early phase of RA by relying on IC formation between anti-CII antibodies and soluble CII fragments, both of which are present in the synovial fluid (Lohmander et al., 2003; Yoshida et al., 2006). In fact, anti-CII IgGs are thought to be locally produced in RA patients as antibody titers are often higher in synovial fluid compared to serum (Lindh et al., 2014; Rowley et al., 1987). **We have previously demonstrated that after i.v. injection in mice, anti-CII mAbs reach the joint and bind cartilage after about 24 hours (Jonsson et al., 1989).** COMP is expressed predominantly in cartilage. Interestingly, **soluble COMP and anti-COMP antibodies are present in synovial fluid of RA patients (Lorenzo et al., 2017; Saxne and Heinegard, 1992; Souto-Carneiro et al., 2001) and** pathogenic anti-COMP mAbs also induced pain-like behavior very early, prior to any signs of inflammation **in mice** after i.v. injection, as well as after i.a. injection in IC formation. **Thus, we speculate that systemic injection of monomeric antibodies that bind antigens in the joint *e*.*g*. soluble CII fragments or COMP, leads to local formation and accumulation of IC, which activates FcγRI on sensory neurons at concentrations that are lower than those required for the induction of inflammation. In line with this hypothesis, when we inject pre-formed IC intra-articularly the mechanical hypersensitivity develops faster compared to i.v. injection of monomeric antibodies (hours compared to days), still in the absence of visual signs of inflammation. Furthermore, we only observed neuronal responses in DRG cultures when the antibodies were applied as pre-formed ICs, monomeric CII mAbs were without effect as the antigen is not present in our in vitro system, and thus, there is no IC formation.** Thus, while the antigen is critical for IC formation, the IC-FcγR interaction on sensory neurons may represent a more general pain mechanism.

We have previously shown that human IgG is not detectable in the spinal cord 7 days after i.v. injection to naive mice (Wigerblad et al., 2016). Thus, the primary site of the pronociceptive actions of anti-CII and anti-COMP antibodies are most likely peripheral rather than central. However, prolonged activation of primary afferents often leads to spinal sensitization, an important component of pain chronicity. While outside the scope of the current work, future studies exploring such aspects of neuronal FcγR-mediated hypersensitivity are important

While FcγRs expression in rat DRGs has been explored, very little information is available with regards to their neuronal expression and associated pro-nociceptive function in mice. Thus, we carefully mapped mRNA and protein expression of the FcγRs in mice and found that *Fcgr1*, *Fcgr2b* and *Fcgr3* mRNA was readily detectable in mouse DRG, both in the soma of sensory neurons and in non-neuronal cells. In naive rats, only FcγRI is present in DRGs and protein expression is detectable exclusively in neurons (Qu et al., 2012; Qu et al., 2011). In contrast, we found both FcγRI and FcγRIIb protein in naive mouse DRGs; a finding that we confirmed using different techniques. Strikingly, mouse FcγRI protein expression was not detectable in the soma of DRG neurons, but instead was seen in resident DRG macrophages. FcγRIIb, on the other hand, was present in neuronal cell bodies. While the expression pattern of FcγRI has not been previously explored in the mouse sensory nervous system, expression in motor neurons (Mohamed et al., 2002) has been suggested

The surprisingly high number of *Fcgr1* mRNA molecules in DRG fiber tracts caused us to hypothesize it may be axonally transported for local translation. Indeed, the machinery for mRNA translation can be found along the sensory axons where local translation has been shown to regulate peripheral nociceptor plasticity (Jimenez-Diaz et al., 2008; Obara et al., 2012; Price and Geranton, 2009). By being locally translated, proteins are believed to display more specific targeting. While further work is necessary to determine if FcγRs are locally translated in nociceptors, we did find that ligation of the sciatic nerve resulted in accumulation of *Fcgr* mRNA molecules proximal to the ligature site and protein expression of FcγRI, as well as FcγRIIB, is present in nerve fibers in the skin. Thus, it is an intriguing possibility that changes in neuronal FcγR expression may be a specific regulatory modulation in response to injury or inflammation, allowing for an increased capacity to react to ICs by enhanced neuronal excitability.

Previous work showed that intra-plantar injection of OVA-ICs induces pain-like behavior (Jiang et al., 2017). Bringing this into the context of arthritis in the joint we show that intra-articular injection of CII-IC, COMP-IC and OVA-IC rapidly induced mechanical hypersensitivity in WT mice, but CII-IC failed to do so in FcRγ-chain-/- mice. Even though the site of neuronal synthesis differs between mouse and rat, our studies indicate that also in naive mice, FcγRI is present at peripheral neuronal terminals, enabling the sensory nervous system to respond directly to the presence of ICs.

Our experiments using modified anti-CII antibodies that retain their ability to bind CII but either lack the Fc region or have a reduced affinity for FcγRs, and FcRγ-chain-/- mice, showed that the Fc-FcγR interaction is critical for development of CII-antibody-induced pain-like behavior. These experiments do not rule out that pain-like behavior is the consequence of IC activation of inflammatory cells. However, the lack of FcγRI expression on satellite cells and the fact that CII-IC failed to induce CGRP-release in cultures generated from FcRγ-chain-/- mice, but still increased intracellular [Ca2+] signal in cultures from FcγRIII-/- mice, strongly suggest that CII-IC can act on FcγRI on sensory neurons and drive neuronal excitation. Furthermore, previous work showed that mast cells are not involved in this process in rats (Jiang et al., 2017) and we found that mice depleted of activating FcγR in myeloid cells still developed early CAIA hypersensitivity. Conversely, mice lacking activating FcγR on non-myeloid cells, including neurons, were protected from developing mechanical hypersensitivity after injection of anti-CII antibodies. Thus, accumulating evidence points to a critical role of neuronally expressed FcγR in the induction of pain prior to inflammation.

The expression pattern of FcγRs in human DRGs was hitherto unexplored. Interestingly, in accordance with our observations in mice, FcγRI protein expression is not localized to neuronal cell bodies but to non-neuronal cells in human DRGs, which based on morphology and localization appear to be resident macrophages. Further work is warranted to determine if FcγRI is transported and locally translated in humans. Noteworthy, we found protein expression of another activating FcγR, FcγRIII, in human DRG NeuN positive neurons. Although the FcγRIII antibody used does not differentiate between FcγRIIIA and FcγRIIIB, we did not detect any signal with antibodies specific for FcγRIIIB, and as *FCGR3A* mRNA levels are 30-fold higher than *FCGR3B* in human DRGs. Thus, FcγRIIIA is an interesting target for further human studies. Considerable cell-type specific expression profile differences exist between mouse, rat and human FcγRs, which certainly calls for caution when interpreting the translational potential of these data. Nevertheless, it is an intriguing possibility that human sensory neurons also respond to ICs through similar neuronal-*FCGR pathways* as FcγR’s represent a novel potential therapeutic target for pain in conditions with an autoimmune component.

In summary, the present study supports a novel view on how autoantibodies can act as pro-nociceptive factors. Local formation of soluble ICs has the potential to serve important roles in both the induction and maintenance of pain via mechanisms mediated by direct interactions between ICs and neuronally expressed FcγRI. This study shows that CII-ICs, which are highly correlated with early RA and joint pathology, serve as key triggers for pain-behavior in the early phase of the disease. Indeed, our studies point to a functional coupling between autoantibodies and pain transmission, even in the absence of inflammation, and open new avenues for decoding pain mechanisms in autoimmune diseases.

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# The authors declare the subsequent conflicts of interests. Hansa Medical AB (HMAB) ([www.hansamedical.com](http://www.hansamedical.com/)) holds patents for using EndoS as a treatment for antibody-mediated diseases. MC is listed as one of the inventors on these patents and has a royalty agreement with HMAB. MC also holds patents for the biotechnological use of EndoS.

# Author Contributions

Conceptualization, ABF, GW, DN, DBB, KSN, LD, SC, RH and CIS; Methodology, ABF, GW, DN, DBB, K.S.N., KS, BX, LEB, FW, BH, MCo, KK, BH, JMA-J, SC; Investigation, ABF, GW, DN, DBB, CMU, KS, SA, KÄM, AB, LZ, JS, LEB, KK, JMAJ, JL, SC; Resources, KSN, BX, MC, A-JC-D, LD, IB, KK, MCo., FW, BH, RH and CIS; Writing – Original Draft, ABF, GW, DN, DBB, KSN, MH, RH and CIS; Writing – Review & Editing, all authors; Supervision, KSN, KJ, LD, JTL, KK, SC, RH and CIS; Funding Acquisition, RH and CIS.

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**Figure legends**

**Figure 1. Injection of anti-CII antibodies induces pain-like behavior prior to visual, histological and molecular signs of arthritis.**

(A, B and C) **B10.RIII mice** injected with anti-CII mAbs (n=19; saline controls n=17) started developing joint inflammation around day 6 (A). Day 9 all animals displayed signs of arthritis (B). Mechanical hypersensitivity (C) was observed already on days 3 and 5, prior to onset of arthritis, and persisted throughout day 21.

(D) Representative H&E histology of **B10.RIII** mouse ankle joints collected 5 and 15 days after injection of anti-CII mAbs. While an inflammatory infiltrate, bone erosion and cartilage serration were visible day 15, no signs of joint pathology was detectable day 5 or in saline controls.

(E, F and G) Scores for inflammatory hallmarks as synovitis (E), bone destruction (F), and loss of cartilage (G) revealed mild ankle joint pathology in 2 of 8 mice day 5 and prominent signs in all mice day 15 (n=5). Control mice represent pooled time-matched saline injected mice (n=4+4).

(H-I) qPCR analysis of joint extracts showed a significant increase in mRNA levels of most of the inflammatory factors investigated at day 15 (n=7) while none of them were elevated at day 5 CAIA (n=6), when compared to saline controls (n=5) (H-I).

(J-K) Activation of MMPs was only significantly increased after 15 days of CAIA, while no changes were detected at day 5 (n=3/group, **B10.RIII mice**).

Data are presented as mean ± S.E.M. \* = P < 0.05; \*\* = P < 0.01; \*\*\* = P < 0.001 compared to saline controls.

**Figure 2. Anti-CII antibodies injected either as a cocktail or as individual antibodies induce mechanical hypersensitivity and reduce locomotion prior to inflammation.**

(A, B and C) Anti-CII mAbs (n=10) induced mechanical hypersensitivity as early as 2 days post-injection (A) compared to saline controls (n=9) in **B10.RIII** mice. Arthritis scores (B) and incidence (C) were not detectable until day 4, and remained very low also day 5.

(D) Total movement (D-left) and rearing (D-right) significantly decreased in **B10.RIII mice** injected with the anti-CII mAb cocktail (n=15), compared to controls (n=19).

(E, F and G) When injected individually, the four mAbs (M2139, UL1, CIIC1, CIIC2) induced mechanical hypersensitivity similarly to the cocktail (E) (n=5-9/group, **B10.RIII mice**). No considerable signs of inflammation (F-G) were detected.

(H) Total movement (H-left) and rearing (H-right) were reduced in M2139 mAb injected **B10.RIII mice** (n=5), compared to saline (n=5) or isotype control (n=5).

(I, J and K) Injection of M2139 mAb induced mechanical hypersensitivity for 21 days (I) even at doses that did not induce visual signs of inflammation (J-K) (n=5, **B10.RIII mice**).

**Axes in Fig. 2A and 2E are interrupted in order to make the difference between groups clearer to visualize.**

Data are presented as mean ± S.E.M. \* = P < 0.05; \*\* = P < 0.01; \*\*\* = P < 0.001 compared to saline controls.

**Figure 3. Anti-CII antibody-induced pain-like behavior is not mediated by complement activation or cartilage destruction. Non-arthritogenic anti-CII mAb still elicits mechanical hypersensitivity.**

(A) Injection of the C5a-receptor antagonist PMX53 (C5aR ant) (n=5) did not prevent anti-CII mAbs-induced mechanical hypersensitivity (n=4, B10.RIII mice) compared to vehicle (saline) injected controls (n=7, **B10.RIII mice**).

(B and C) Antagonizing the C5a-receptor (n=5, **B10.RIII mice**) did not prevent anti-CII mAbs-induced reduction in total movement (B) and rearing (C) compared to saline controls (n=19, B10.RIII mice).

(D, E and F) Complement 5 deficient (C5-/-) mice developed mechanical hypersensitivity (n=5) and displayed a reduction in total movement (E) and rearing (F) (n=4) comparable to WT **B10Q** mice (D) (n=6-8) after injection of anti-CII mAbs.

(G) **B10.RIII** mice injected with the non-arthritogenic CIIF4 antibody (n=8) developed mechanical hypersensitivity from day 3 post-injection compared to saline controls (n=7).

Data are presented as mean ± S.E.M. \* = P < 0.05; \*\* = P < 0.01; \*\*\* = P < 0.001 compared to saline controls.

**Figure 4. FcγRs are expressed in mouse DRG neurons.**

(A) Microarray data showed mRNA for *Fcgr1-4* in **B10.RIII** mouse DRG (n=3).

(B)qPCR showed mRNA for *Fcgr1-4* in **B10.RIII** mouse DRG (n=10).

(C) Publicly available RNA-seq of **C57BL/6 mouse DRGs** show the presence for *Fcgr1-4* (n=3).

(D) smFISH showed mRNA molecules for *Fcgr1*, *Fcgr2b* and *Fcgr3* in **BALB/c** mouse DRG co-localizing with NeuN. Scatter graph shows number of mRNA molecules in neuronal soma.

(E) FcγRI protein expression was detected by WB in DRGs from WT **BALB/c** mouse, but not from FcRγ-chain-/-mice.

(F) Proteomic analysis identified peptides specific for FcγRIIb in **BALB/c** mouse DRG.

See also Figure S1 & S4.

**Figure 5. FcγRI and FcγRIIb are expressed in the DRG and in nerve fibers in the skin.**

(A and B) FcγRI immunoreactivity was detected in WT **BALB/c DRGs**, but not in FcRγ-chain-/-mice (A), co-localizing with Iba1 positive resident macrophages (B).

(C and D) FcγRIIb immunoreactivity was detected in **BALB/c** mouse DRG and retained in FcRγ-chain-/-mice (C), co-localizing with TrkA positive neurons (D).

(E and F) FcγRI (E) and FcγRIIb (F) immunoreactivity was detected in PGP9.5 positive nerve fibers in **BALB/c** mouse glabrous skin.

(G, H and I) smFISH on **BALB/c** mouse sciatic nerves after ligation (G) revealed accumulation of mRNA molecules for *Fcgr1* (H) and *Fcgr2b* (I) proximal to the site of ligation (Ipsilateral), while barely any signal was found in the contralateral intact nerve.

See also Figure S2, S3 & S4.

**Figure 6. CII-IC stimulation of DRG cell cultures leads to increased neuronal excitability.**

(A) FcγRI and FcγRIIb are expressed in **BALB/c** mouse DRG neurons in culture as shown by co-localization with βIII-Tubulin.

(B and C) CII-IC stimulation of **BALB/c** mouse DRG cell cultures resulted in increased intracellular [Ca2+] signal (B) and also evoked positive inward currents (C).

(D and E) CII-IC stimulation evoked CGRP release in DRG cell cultures from WT **BALB/c** mice (D) but not from FcRγ-chain-/- mice (E).

Data are presented as mean ± S.E.M. \*\*\* = P < 0.001.

**Figure 7. Different ICs promote pain-like behavior *in vivo* and FcγRIV-/- mice develop mechanical hypersensitivity despite lack of collagen antibody induced arthritis**

(A and B) Intra-articular injection of CII-IC induced mechanical hypersensitivity in WT **BALB/c mice** (n=14-21/group) (A) but not in FcRγ-chain-/- mice (n=8-10/group) (B).

(C and D) Systemic administration of anti-CII mAbs evoked mechanical hypersensitivity in WT **BALB/c** mice (n=8/group) (C) but not FcRγ-chain-/- mice (n=8/group) (D).

**(E) Intra-articular injection of IgG-IC induced mechanical hypersensitivity in WT BALB/c mice (n=6/group).**

**(F) Intra-articular injection of COMP-IC induced mechanical hypersensitivity in WT C57BL/6 mice (n=7/group).**

**(G and H) Systemic administration of anti-COMP mAb evoked mechanical hypersensitivity in WT BALB/c mice (n=5, G), in the absence of any signs of inflammation (H).**

(I, J and K) Systemic injection of anti-CII mAbs induced pain-like behavior in both WT C57BL/6 mice (n= 4, I) and FcγRIV-/- mice (n=6, J), even if in the latter no signs of inflammation were observed (K).

**Axes in Fig. 7A-D are interrupted in order to make the difference between groups clearer to visualize.**

Data are presented as mean ± S.E.M. \* = P < 0.05; \*\* = P < 0.01; \*\*\* = P < 0.001 compared to saline/PBS controls.

**Figure 8. The pronociceptive properties of anti-CII antibodies are dependent on the Fc region, glycosylation and interaction with FcγRI in the joint.**

(A, B and C) **B10.RIII mice** injected with anti-CII mAb Fab fragments (n=8) did not develop mechanical hypersensitivity (day 5, A) compared to CAIA (n=13) and controls (n=7) mice. They also did not show reduction in total movement (B) or rearing (C) (night 3, n=8-19/group).

**(D) B10.RIII mice injected with EndoS treated anti-CII mAbs did not develop mechanical hypersensitivity (n=3/group)**

(E, F and G) **B10.RIII mice** injected with EndoS treated anti-CII mAb M2139 did not develop mechanical hypersensitivity (day 5, E) or display a reduction in locomotor activity (F-G) (night 3, n=6-7/group).

(H, I and J) **BALB/c mice** lacking activating FcγRs in myeloid cells (KO-WT) (I) developed mechanical hypersensitivity after injection of anti-CII mAbs as compared to controls (WT-WT) (J). In contrast, mice lacking activating FcγRs in non-myeloid cells (WT-KO), including neurons, were protected (H) (n=8-9/group)**.**

Data are presented as mean ± S.E.M. \* = P < 0.05; \*\* = P < 0.01; \*\*\* = P < 0.001 compared to controls.

**Figure 9. FcγRI and FcγRIII are expressed in human DRG.**

(A) Publically available data show the presence of *Fcgr* mRNA in human DRGs (n=6). *Fcgr3a* is the highest expressed.

(B and C) FcγRI immunoreactivity was detected in human DRGs **(n=4)**. The lack of co-localization with NeuN and the morphology of the FcγRI positive cells suggest FcγRI expression in resident macrophages, similarly to mice. White arrows indicate FcγRI positive cells, which are negative for NeuN.

(D and E) Immunoreactivity for the activating FcγRIII in human DRGs **(n=4)** co-localized with the neuronal marker NeuN. White arrows point to double positive neurons (FcγRIII and NeuN).

**Supplementary figures legends**

**Figure S1 *(Related to Figure 4)*.** Quantification of mRNA molecules of *Fcgr1*, *Fcgr2b* and *Fcgr3* plotted per individual cell according to neuronal area and showed as intensity of color gradient. *Fcgr1* is shown expressed at the highest level.

**Figure S2 *(Related to Figure 5)*. A and B)** IHC show lack of co-localisation between FcγRI staining and vimentin and TrkA. **C and D)** No immunoreactivity for FcγRIII and FcγRIV was detected in **BALB/c** mouse DRG. **E)** FcγRI immunoreactivity is present in resident macrophages of **BALB/c** mouse DRG. **F, G and H)** **Antibodies from 3 different sources (Dr. Mark Cragg, Sinobiologicals and R&D Systems) showed FcγRI immunoreactivity in resident macrophages in DRGs from C57BL/6 mice.**

**Figure S3 *(Related to Figure 5)*. A)** FcγRI immunoreactivity is present in neuronal cell bodies of **BALB/c** mouse DRG when using an antibody from Santa Cruz, but the signal is retained in DRGs from FcRγ-chain-/- mice, indicating non-specific binding. **B, C and D)** Antibodies from 3 different sources (Dr. Mark Cragg, Sinobiologicals and R&D Systems) showed FcγRI immunoreactivity in resident macrophages **in BALB/c mouse DRGs** and the signal is absent in DRGs from FcRγ-chain-/- mice, indicating specific binding.

**Figure S4 *(Related to Figure 4 & 5)*.** smFISH in mouse DRGs showed *Fcgr1*, *Fcgr2b* and *Fcgr3* mRNA molecules to be expressed the highest in fiber tracts (non-nuclear and non-neuronal areas).

**Material and methods**

**Animals**

All the experiments were approved by the local ethics committee for animal experiments in Sweden (Stockholm Norra Djurförsöksetiska nämnd). For some experiments, BALB/c and C57BL/6 mice were purchased from Charles River and Janvier Laboratories The B10.RIII, B10Q strains, B10Q.C5\*transgenic mice (mice with congenic 2-bp deletion in the complement 5 encoding gene making it nonfunctional) (Johansson et al., 2001), FcγRIII-/- (founder from the Jackson Laboratories) (Hazenbos et al., 1996) and FcγRIV-/- (Nimmerjahn et al., 2010) mice were bred at the Karolinska Institutet. FcγRIII-/- mice and C5\* mice were bred on B10Q background and FcγRIV-/- mice on C57BL/6 background for more than 10 backcrosses. BALB/c WT and FcRγ chain-/- mice (lacking the activating receptors FcγRI, III and IV) (Takai et al., 1994) were backcrossed for 12 generations to Balb/c and bred at the National Veterinary Institute, Uppsala, Sweden. **For experiments involving transgenic mice we used homozygous WT littermates mice as controls, except for experiments with FcRγ chain-/- mice. The WT control and FcRγ chain-/- mice originate from the same breeding line but were maintained as homozygous mice in parallel.** Both male and female mice 12-20 weeks of age were used and all mice were housed in standard cages (3-5 per cage) in a climate-controlled environment maintaining a 12 h light/dark cycle with access to food and water ad libitum. This study conforms to the ARRIVE guidelines.

**Antibodies and drugs**

The antibody cocktail used for induction of collagen antibody-induced arthritis (CAIA) contains equal amounts of 4 arthritogenic anti-CII mouse monoclonal antibodies (mAbs): M2139 (IgG2b, J1 epitope), CIIC1 (IgG2a, C1 epitope), CIIC2 (IgG2b, D3 epitope), and UL1 (IgG2b, U1 epitope) (Nandakumar and Holmdahl, 2005). The anti-CII mAb CIIF4 was used as a non-arthritogenic CII binding antibody (Croxford et al., 2010; Nandakumar et al., 2008). mIgG2a (mouse anti-human HLA-DRa [L243], Abcam) and mIgG2b (mouse anti-human parathyroid epithelial cells) were used as isotype control mAbs. 15A11 was used as anti-COMP mAb (Geng et al., 2012). Antibodies were produced and purified as described earlier (Nandakumar and Holmdahl, 2005). Collagen II mAb immune complex (CII-IC) stock solution (1 mg/ml) was prepared by mixing anti-CII mAb cocktail (1 mg/ml) with rat CII (1 mg/ml), at a ratio of 1:1 at 37°C with gentle shaking for 30 min (Burkhardt et al., 2002). Similarly, COMP-IC and IgG-IC were prepared by mixing antibodies (respectively 15A11 anti-COMP and mouse anti-rat IgGs) with antigen (respectively COMP and rat IgGs) at a ratio respectively of 6:1 and 1:1 at 37°C with gentle shaking for 1 hour.

To **hydrolyze** the N-linked Fc-glycans, M2139 mAb was incubated with recombinant endo-β-N-acetylglucosaminidase (EndoS) fused to glutathione S-transferase (GST) as previously described (Collin and Olsen, 2001). Briefly, GST-EndoS in phosphate buffer solution (PBS) was mixed with M2139 mAb **or anti-CII mAbs cocktail** and incubated at 37°C for 16 h. GST-EndoS was then removed using Glutathione-Sepharose 4B columns (GE Healthcare). Further purification of the antibodies was done using ion exchange column. SDS/PAGE and Lens culinaris agglutinin (LCA) lectin blotting were used to confirm complete removal of GST-EndoS and efficacy of EndoS cleavage. Fab fragments were prepared from the anti-CII mAb cocktail using the Pierce Fab Preparation Kit (Thermo Scientific) according to the manufacturer’s instructions.

**Experimental models and drug/antibody administration**

All mAbs were injected intravenously (i.v.). CAIA was induced by injection of anti-CII antibody cocktail (4 mg in 150 µl saline) followed by intra-peritoneal (i.p.) injection of lipopolysaccharide (E.Coli LPS, 25 μg in 100 µl saline, 055:B5, Sigma) 5 days later. LPS boosts the immune activity and synchronizes the onset of disease,

detectable as a rapid increase in the arthritis score and incidence of arthritis. In the experiment with FcγRIV-/- mice, a 5-clone CII antibody cocktail was injected i.v. (3 mg, Chondrex) followed by 50 μg LPS in 100 µl saline (055:B5, Sigma) day 5 as this gives a higher arthritis incidence in C57BL/6 mice. The early phase of the CAIA model was defined as day 0-5 after injection of anti-CII antibodies (i.e. prior to LPS injection). CIIF4, M2139, CIIC1, CIIC2, UL1 and control IgGs were injected individually (4 mg in 150 µl saline). Also, Fab fragments and EndoS treated antibodies corresponding to 4 mg anti-CII mAb cocktail were injected i.v in 150 µl saline. **For different doses test**, 0.5 to 4 mg of M2139 mAb was administered in 150 µl saline. The cyclic peptide C5a-receptor inhibitor (PMX53, 3 mg/kg in saline) (Academia Sinica, gift from Dr. Alice Yu, UCSD, California) was injected subcutaneously (s.c.) 1 h prior to injection of anti-CII mAb cocktail and then once daily 3 h prior to assessment of mechanical hypersensitivity.

For intra-articular (i.a.) injections mice were anesthetized with isoflurane (induction: 5%, maintenance: 2.5%) and different ICs (500 ng in 5 µl PBS) injected into the ankle joint using a 29G needle. For bone marrow (BM) transplantation experiments, recipient BALB/c Fcγ chain-/- and WT mice were irradiated with 750 rad. The following day, BM cells were harvested from donor mice by flushing the tibia and femur and 10x106 cells in 0.2 ml PBS were injected i.v. to the recipient mice. Irradiated FcRγ-chain-/- mice received WT BM cells, which generated mice with activatory FcγR expression on hematopoietic cells but negative on other cells including neurons (WT-KO). Irradiated WT mice received BM from FcRγ-chain-/- mice, which generated mice with activating FcγRs only on non-hematopoietic cells including neurons (KO-WT). Irradiated WT mice receiving BM cell transfer from WT mice (WT-WT) were used as controls. Recipient mice were injected with saline or anti-CII mAbs i.v. (4 mg) 6 weeks after irradiation. Nerve ligation was established by ligating the tibial and common peroneal branches (with 6-0 silk suture) under isoflurane anesthesia. Mice received buprenorphine (0.1 mg/kg s.c.) every 12h for the following 2 days after surgery.

**Assessment of arthritis**

The development of arthritis in the fore and hind paws was monitored by visual inspection as described previously (Bas et al., 2012; Holmdahl et al., 1998). Briefly, visible signs of inflammation, defined as redness and swelling, were scored on a 0–60 scale by investigators blinded for the origin and treatment of the mice. Each inflamed digit was noted as 1 point and inflammation of the metacarpus/metatarsus and ankle joint as 5 points, giving a maximum of 15 points per paw. Incidence was calculated as percentage of mice that were positive for arthritis. The degree of arthritis was also assessed by histology. Mice were deeply anesthetized with volatile isoflurane (5%) and perfused with saline followed by 4% paraformaldehyde (PFA). Hind ankle joints were post-fixed in 4% PFA for 48 h, decalcified in EDTA (Sigma) solution for 4-5 weeks and then dehydrated in ethanol and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E, Histolab) and scored by blinded investigators as previously described (Bas et al., 2012) on a scale from 0-3, where 0 is normal and 3 is severe synovitis, bone erosion, and/or cartilage destruction.

**Assessment of pain-like behavior**

Mechanical hypersensitivity in the hind paws and reduced locomotion were used as measures of evoked and spontaneous pain-like behavior, respectively. Assessment of mechanical hypersensitivity was performed on indicated days and locomotion was assessed during the night between day two and three (third night) after antibody injection. The investigators were blinded to the origin and treatment of the mice during behavioral assessments and data analysis.

***Mechanical hypersensitivity***Paw withdrawal thresholds were measured using von Frey filaments. Mice were habituated to the testing cages; individual compartments on top of a wire-mesh surface (Ugo Basile), prior to baseline recordings. On test days, mice were habituated to the test environment for 1 h prior to testing. Withdrawal thresholds were assessed by application of OptiHair filaments (Marstock OptiHair) of increasing stiffness (0.5, 1, 2, 4, 8, 16, and 32 mN, corresponding to 0.051, 0.102, 0.204, 0.408, 0.815, 1.63 and 3.26 g, respectively) to the plantar surface of the paw. A brisk withdrawal of the paw from the filament within 2-3 seconds was noted as a positive response. The 50% withdrawal threshold (force of the filaments necessary to produce a reaction from the animal in 50% of the applications) was calculated using the Dixon up-down method (Chaplan et al., 1994) and expressed in grams. Results from both hind paws were averaged. Assessment of mechanical hypersensitivity was performed between 10:00 – 17:00.

***Locomotion***Locomotor activity was measured using a Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments). Mice were acclimatized to the cages and individual housing for 24 h prior to a 12 h period (18.00-06.00) of automated recordings every 20 min. Movements in the X, Y and Z axes were monitored during the third night after injection of antibodies, by recording the number of infrared beam breaks. The data is presented as total movement (total number of XY-axis beam breaks) and rearing (number of beam breaks in the Z-axis) either over time or accumulated during the 12 h period. One or two control mice were included in each run and the reference (control) group accumulated over the course of the locomotor experiments.

**Inverted grid test**

**Grip and muscular strength and forced movements were assessed by placing the mice on a surface (grid), which is then gradually turned upside-down. The turning takes 10 seconds, and then the latency to the mice loosing their grip and falling off the grid is measured (with a cut off of 10 seconds). The inverted grid test was performed 5 days after injection of saline or anti-CII mAbs. The investigators were blinded to the origin and treatment of the mice during the behavioral assessment.**

**Metalloprotease activity**

Mice injected with either saline or CII mAb cocktail received i.v. injection of MMPsense 680 (Galligan and Fish, 2012) (2 nmol in 150 μl PBS, PerkinElmer), an optically inert dye that becomes fluorescent in the presence of active MMPs, 24 h before sacrifice by decapitation.  Paws were removed and scanned with Odyssey CLx (LI-COR) and signal intensity is presented normalized to saline injected mice.

**Microarray expression analysis**

Lumbar DRGs (L3-5) were dissected and total RNA extracted and purified using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA concentration was measured using an ND-1000 spectrophotometer (NanoDrop Technologies) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies). Total RNA (250 ng) from each sample was used to generate amplified and biotinylated sense-strand cDNA from the entire expressed genome according to the Ambion WT Expression Kit (P/N 4425209 Rev C 09/2009) and AffymetrixGeneChip® WT Terminal Labeling and Hybridization User Manual (P/N 702808 Rev. 5, Affymetrix Inc.). GeneChip® ST Arrays (GeneChip® Mouse Gene 2.0 ST Array) were hybridized for 16 h in a 45°C incubator, rotated at 60 rpm. According to the GeneChip® Expression Wash, Stain and Scan Manual (PN 702731 Rev3, Affymetrix Inc.) the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip® Scanner 3000 7G. The raw data was normalized in the free software Expression Console provided by Affymetrix ([http://www.affymetrix.com](http://www.affymetrix.com/)) using the robust multi-array average (RMA) (method first suggested by Li and Wong, 2001), followed by extraction of the expression levels of the genes of interest (*Fcgr1, Fcgr2b, Fcgr3* and *Fcgr4*). Data are presented using a log2 scale with expression cutoff at 5,5.

**Quantitative real-time polymerase chain reaction (PCR)**

Mice were decapitated under volatile anesthesia and ankle joints collected fresh, trimmed from muscle and tendons, snap frozen and stored at -70 °C. For RNA extraction, joints were then pulverized using BioPulverizer (BioSpec) and briefly sonicated in TRIzol (Invitrogen) using ultrasonic processor (EW-04714-51, Cole Parmer).

RNA was extracted according to manufacturer’s protocol and reverse transcribed to complementary DNA. For lumbar DRGs (L3-5), RNA extracted for microarray assay (described above) was used for reverse transcription. Quantitative real-time PCR (Applied Biosystems) was performed with hydrolysis probes,  according to the manufacturer's instructions, to determine the relative messenger RNA (mRNA) levels. Pre-developed specific primer/probe sets for mouse chemokine Ccl2 (Mcp-1, Mm00441242\_m1), inflammatory cytokines Tnf (Mm00443258\_m1), Il1b (Mm00434228\_m1), Il6 (Mm00446190\_m1), mast cell proteases Mcpt4 (Mcp-4, Mm00487636-g1), Tpsb2 (Mcp-6, Mm01301240\_g1), pro-inflammatory enzyme Cox2 (Mm00478374\_m1), matrix metalloproteases Mmp2 (Mm00439498\_m1), Mmp9 (Mm00442991\_m1), Mmp13 (Mm00439491-m1), and reference gene Hprt1 (Mm01545399\_m1) (all from Applied Biosystems) were used to determine threshold cycle values to calculate the number of cell equivalents in each sample with the standard curve method (Boyle et al., 2003). Data were normalized to Hprt1 values and expressed as relative expression units (REU).

**Single-molecule fluorescence in situ hybridization (smFISH)**

smFISH was carried out as previously described with some modifications (Codeluppi et al., 2018). Mice were perfused with PBS under isoflurane anesthesia, DRGs and sciatic nerves collected and frozen in OCT. After cryosectioning (10 µm), the sections were post-fixed in 4% PFA (10 min at room temperature) and stored at -80 °C until use. For hybridization, the sections were first permeabilized for 10 min with methanol in -20 °C, cleared with 4% SDS and after heat shock at 70C for 10 min incubated with 250 nM of fluorescent labeled probes for 4h (Biosearchtech) at 37 °C. After imaging the tissues were counterstained with DAPI (Thermo) and NeuN-Alexa 488 conjugated antibody (Millipore, ABN78A4) for the specific labeling of neuronal cell bodies and TrkA antibody (R&D systems, AF1056) to visualize axons. Sections were mounted with Pro-long Gold (Thermo) and image stacks (0.3 μm) were acquired using a customized scanning microscope (Nikon TE). Images were analyzed using a custom Python script (pysmFISH python package). After background removal, a laplacian-of-gaussian was used to enhance the RNA dots that were defined as the local maxima above a threshold automatically calculated, after removal of connected components larger than dots. Quantification of single mRNA molecules as well as the cell diameter was done manually using Image J™ and data were plotted as number of RNA molecules according to cell size. The number of mRNA molecules per cell was then translated into a color gradient map and plotted according to cell size.

**Proteomic analysis**

Lumbar DRGs (L3-L5) collected from BALB/c mice were lysed by bead beating (Tissue Lyser, Qiagen) in TEAB buffer (Sigma-Aldrich) followed by protein thiol reduction. DRG lysates were digested to peptides using a Filter-aided sample preparation (FASP) method essentially as described (Wisniewski et al., 2009) with minor modifications (Wisniewski, 2016). The resulting peptides were recovered and proceeded to analysis (labeled and label-free). Peptide digests from one animal were labeled chemically using Tandem Mass Tag 6-Plex reagents according to the manufacturer’s instructions (Thermo Scientific) and the remaining peptide pool was analyzed unlabeled. To increase the proteome coverage, 100 µg of either TMT labeled or unlabeled peptide samples were pre-fractionated using high pH reverse phase liquid chromatography. The fractions were evaporated, reconstituted in 0.1% FA and analyzed by high resolution nanoLC-MS/MS on Q-Exactive Orbitrap mass spectrometers (Thermo Scientific) coupled to high performance nano LC systems (Dionex Ultimate-3000, Thermo Scientific, Sunnyvale CA, USA) set up in a trap and elute configuration. For the TMT labeled sample pool, data was also acquired using an identical nanoLC system interfaced to a TriBrid Orbitrap based mass spectrometer (OrbiTrap Fusion, Thermo Scientific).

**DRG cell culture**

DRGs (C1-L6) from WT and Fcγ chain-/- mice were extracted and placed in ice-cold Dulbecco’s PBS (DPBS) until enzymatically dissociated with papain (1.7 mg/ml) (30 min at 37 °C) followed by treatment with collagenase I (2 mg/ml) and dispase II (8 mg/ml) (Sigma) enzyme mixture (30 min at 37 °C). The cells were then gently triturated in Leibovitz’s medium (L15) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin and streptomycin (Invitrogen) and 10 M mitotic inhibitor (5-fluoro-2-deoxyuridine, Sigma). For CGRP release experiments, the nerve growth factor (30 ng/ml, Sigma) was added into the medium. The cell suspension was plated on uncoated well plates for 1.5-2 h before transferred to poly-D-lysine and laminin (Sigma) pre-coated well plates. The cells were maintained at 37 °C in 5% CO2atmosphere and the medium was replaced after 24 h, followed by changes every third day.

**CGRP release**

After 6 days in culture, the medium was removed and the cells were washed twice with HEPES buffer (25 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 3.3 mM dextrose, 0.1% (w/v) bovine serum albumin, pH 7.4 with NaOH) and placed in new HEPES buffer for 30 min at 37 °C (pre-stimulation). The HEPES buffer was collected for analysis of basal CGRP release. The cells were then incubated with CII-IC (0.1, 1 and 10 µg/ml), anti-CII mAb cocktail (1 µg/ml), CII antigen (1 µg/ml), or control IgG2b (1 µg/ml) in HEPES buffer or HEPES buffer alone for 30 min at 37 °C (post-stimulation) and the supernatant was collected for CGRP analysis. Capsaicin (50 nM, Sigma) in HEPES (10 min at 37 °C) was used as a positive control. CGRP levels (pg/ml) in the supernatants were determined with enzyme immune assay (EIA) kit in accordance with the manufacturer's instructions (SPI-Bio, Bertin Pharma). The % change between pre and post-stimulation was calculated for each well.

**Calcium imaging**

After 24 and 48 h in culture, the cells were loaded with Fluo-3 (4.4 µM, Life Technologies) for 30-40 min at room temperature (20–22 °C). The cells were washed with modified HEPES buffer (145 mM NaCl, 3 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM glucose and 10 mM HEPES, pH 7.4 with NaOH) and then placed in the recording chamber and continuously perfused with bath solution (modified HEPES buffer) at a constant flow rate (1 ml/min). Calcium imaging was performed using a Nikon Diaphot inverted microscope with a diode laser (Cobolt dual calypso, Cobolt AB) 488 nm excitation and a 40X oil immersion objective. The change in emission (506 nm) i.e. intracellular calcium bound to Fluo-3 was recorded every 15 s using a PMT (BioRad MRC 1024). CII-IC (1 µg/ml) or control IgG2b (1 µg/ml) was applied for 3 min to the same cells in random order with a minimum of 10 min wash period between applications. At the end of each experiment 50 mM KCl was applied for 1 min to detect functional neurons. All the reagents were prepared from stock solutions and dissolved in modified HEPES buffer. Images were analyzed with ImageJ software (National Institute of Health; available at <http://rsb.info.nih.gov/ij>). In each image, capturing an average of 10 cells, all visible cells were chosen for analyses. Mean fluorescence intensity (F) for the region of interest (ROI), the cell bodies, was measured in each image. F0 was calculated as the average mean intensity of the first 5 images in each series and the data presented as F/F0. **Cells were considered positive if the fluorescence signal increase was at least 20% compared to baseline and higher than three standard deviations.**

**Electrophysiological recordings**

Whole cell voltage-clamp recordings were conducted in small DRG neurons (15-25 µm in diameter) at room temperature (20–22 °C) within 24 and 48 h of culturing using a patch clamp amplifier (Axo-Patch-200A, Molecular Devices). The recordings were filtered at 1 kHz, sampled at 4 kHz, and analyzed by using Clampex 10.4 software (Molecular Devices). Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus; 1.5 mm outer diameter, 0.86 mm inner diameter) using a vertical puller. The resistance of the patch pipettes was 4–5 MΩ when filled with internal solution (120 mM K+-gluconate, 20 mM KCl, 1 mM CaCl2, 2 mM MgCl2, 11 mM EGTA, 10 mM HEPES, 2 mM NaATP, pH 7.15 with Tris-base). Series resistance was not compensated. DRG neurons were continuously perfused with bath solution (modified HEPES buffer, see Calcium imaging protocol) at a constant flow rate (1-1.5 ml/min). CII-IC (1 µg/ml) and control IgG2b (1 µg/ml) were applied for 1 min and capsaicin (0.5 µM) was applied at the end of each recording for 10 s as a control (4 min wash period between applications). Cells were accepted having a resting membrane potential more negative than -40 mV **and considered positive if the measured current was higher than 20 pA and higher than three standard deviations**. All reagents were prepared from stock solutions and dissolved in modified HEPES buffer and applied via an 8-channel ValveLink 8.2 Controller application system (AutoMate Scientific).

**Western Blot**

For western blot analysis, spleen and lumbar DRGs were harvested, snap frozen and stored in -80 °C until homogenized in lysis buffer. 5-10 μg of protein per well was loaded, separated by gel electrophoresis (4-12% Bis-Tris gel, Invitrogen) and transferred to nitrocellulose membranes. Non-specific binding sites were blocked with 5% non-fat milk and the membranes were probed with primary antibody overnight at 4 °C (FcγRI (0.1 µg/ml, 50086-R001, Sino Biological). After washing, the membranes were probed with secondary antibodies conjugated to horseradish peroxidase (HRP; Dako antibodies) and the chemiluminescense signal (SuperSignal West Pico PLUS, Thermo Scientific 34580) was detected by exposure to X-ray film (Fujifilm). Membranes were then stripped (Re-Blot plus, Millipore) and re-probed with primary antibody against β-actin (Cell Signaling; 3700) as a housekeeping protein reference. Quantification was performed using ImageJ software.

**Immunohistochemistry and immunocytochemistry**

For immunohistochemistry (IHC), mice were deeply anesthetized and perfused with 4% PFA. Glabrous skin of the hind paws and lumbar DRGs were post-fixed in PFA (4 h and 24 h, respectively) and cryoprotected for 48 h in 30% sucrose in 0.01 M PBS at 4 °C. For IHC with the FcγRIIb mAb (Table 1) (Tutt et al., 2015), anesthetized animals were perfused with PBS prior to collection and snap freezing of tissues. Both fresh and perfused tissues were frozen in OCT and stored in -70 °C until cryo-sectioning. Human DRGs (snap-frozen L4-5, collected from brain-dead subjects after asystole, n=2, with the consent of first-tier family members) were collected at the University of Pittsburgh, shipped and kept at -70 °C until embedded in OCT. All procedures were approved by the University of Pittsburgh Committee for Oversight of Research and Clinical Training Involving Decedents and the Center for Organ Recovery and Education, Pittsburgh, PA (<https://www.core.org>). Serial frozen sections of glabrous skin (20 μm) and DRGs (14 μm) were cut using a cryostat (NX-70, Thermo Fisher Scientific) and mounted on superfrost plus glass slides. Fresh tissues were post-fixed with acetone (10 min at 4 °C) immediately after sectioning. Primary cell cultures of DRG were also fixed in acetone (10 min at 4 °C) 6 days after plating and stored in PBS at 4 °C until analysis. Non-specific binding was blocked using 5% of normal serum in PBS (selection of serum dependent on species of secondary antibodies). Incubation with the primary antibodies (Table 1) (Tutt et al., 2015) was performed overnight at room temperature. Anti-TrkA antibody was used for visualization of primary afferents as i) a high percentage of joint nociceptors express TrkA (Mantyh et al., 2011) and ii) this antibody worked both on PFA and acetone fixed tissues. Immunoreactivity was visualized using Alexa-conjugated secondary antibodies (1:300, Invitrogen) or Cyanine (Cy)-conjugated antibodies (1:300, Jackson Laboratories). In human DRG sections, tyramide signal amplification (cy5-TSA kit, NEL705A001KT, Perkin Elmer) was performed using appropriate HRP-conjugated secondary antibodies (following the manufacturer’s instructions). Prolong Gold antifade with DAPI (Life Technologies), was used for cover slipping and images were collected using a confocal microscope (Zeiss LSM800) operated by LSM ZEN2012 (Zeiss) software. Figures were assembled in Adobe Illustrator CS6 (Adobe). To facilitate interpretation of the microscopic images, FcγR expression was always shown in green with other markers depicted in red and DAPI staining in blue, independently of the secondary antibody used (images processed in ImageJ without modification of the capture settings).

**Statistical analysis**

For comparing changes in behavior over time, repeated measures two-way ANOVA was used followed by Bonferroni post-hoc test. For differences in fluorescence, CGRP release, tactile thresholds, and locomotion with three groups or more one-way analysis of variance (ANOVA) was used, followed by Bonferroni post-hoc test. For differences in mRNA levels, tactile thresholds, and locomotion with two groups, Students t-test was used. Arthritis and histological scores were compared using the Kruskal-Wallis test followed by Dunn’s multiple comparison post hoc test, using GraphPad software. P values less than 0.05 were considered significant.