EXTENDED REPORT

Autoantibodies to citrullinated proteins may induce joint pain independent of inflammation

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ABSTRACT

Objective An interesting and so far unexplained feature of chronic pain in autoimmune disease is the frequent disconnect between pain and inflammation. This is illustrated well in rheumatoid arthritis (RA) where pain in joints (arthralgia) may precede joint inflammation and persist even after successful anti-inflammatory treatment. In the present study, we have addressed the possibility that autoantibodies against citrullinated proteins (ACPA), present in RA, may be directly responsible for the induction of pain, independent of inflammation.

Methods Antibodies purified from human patients with RA, healthy donors and murinised monoclonal ACPA were injected into mice. Pain-like behaviour was monitored for up to 28 days, and tissues were analysed for signs of pathology. Mouse osteoclasts were cultured and stimulated with antibodies, and supernatants analysed for release of factors. Mice were treated with CXCR1/2 (interleukin (IL) 8 receptor) antagonist reparixin.

Results Mice injected with either human or murinised ACPA developed long-lasting pronounced pain-like behaviour in the absence of inflammation, while non-ACPA IgG from patients with RA or control monoclonal IgG were without pronociceptive effect. This effect was coupled to ACPA-mediated activation of osteoclasts and release of the nociceptive chemokine CXCL1 (analogue to human IL-8). ACPA-induced pain-like behaviour was reversed with reparixin.

Conclusions The data suggest that CXCL1/IL-8, released from osteoclasts in an autoantibody-dependent manner, produces pain by activating sensory neurons. The identification of this new pain pathway may open new avenues for pain treatment in RA and also in other painful diseases associated with autoantibody production and/or osteoclast activation.

INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic autoimmune disease that clinically is typified by swelling and reduced function in affected joints, and with joint pain as one of the dominant symptoms.[1] A so far unexplained feature of chronic pain in RA is the frequent disconnect between pain and inflammation. Joint pain (arthralgia) often develops before signs of joint inflammation and is thus one of the first indicators of an emerging RA. Interestingly, autoantibodies also frequently occur in the preclinical phase of the disease and can be detected months to years prior to diagnosis.[2–4] Further, recent studies show that despite reduced disease activity in response to treatment with disease-modifying antirheumatic drugs, many patients continue to report mild, moderate or severe pain.[5–7] However, the mechanisms that are responsible for the inflammation-independent pain that occurs both in the very early phase of the disease and in periods of medically controlled disease activity[8] are so far unknown. Here, we address the possibility that certain autoantibodies directed towards post-translational (citrullinated) proteins may drive the non-inflammatory pain associated with RA.

Anticitrullinated protein antibodies (ACPA) are present in a major subset of patients with RA and are used clinically as serological markers for the diagnosis of RA.[9] Citrullinated autoantigens in patients with RA include fibrinogen, vimentin, α-enolase, collagen type II, immunoglobulin-binding protein and histone 4.[10] Although ACPA is associated with arthralgia before the onset of inflammation and a more aggressive RA subsequent to clinical diagnosis, relatively little is known about the potential pathogenic effects of the human ACPA response.[11] It has been suggested that ACPA together with other pathogenic antibodies facilitate the development of experimental arthritis in mice.[12 13] Furthermore, recent evidence show that certain ACPA alone can induce alterations in bone metabolism and bone loss after binding to the cell surface of osteoclasts.[14]

Previous studies on the contribution of autoantibodies to pain have predominantly been concerned with their role in inflammatory processes, leading to activation of inflammatory cells and the subsequent release of pain-inducing cytokines and prostaglandins. Recent work, however, shows that autoantibodies against neuronal voltage-gated potassium channels induce pain without signs of inflammation,[15] suggesting that autoantibodies may in some contexts have a role in chronic pain states also in the absence of apparent inflammation. The aim of the present study was to examine whether ACPA can induce and maintain joint pain, and whether this is coupled to joint pathology.

MATERIALS AND METHODS

Detailed information on materials and methods is available as online supplementary file.

Animals and injections

Experiments were performed using male B10.RIII mice (Department of Medical Biochemistry and Biophysics, Karolinska Institutet) and BALB/c (Harlan) 15–22 weeks of age. 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*. All experiments were approved by the local ethics committee for animal experiments in Sweden (Stockholm Norra Djurförsöksetiska nämnd). Mice were injected intravenously with either saline or human IgG (hACPA and controls 0.125–4 mg), monoclonal murinised ACPA antibodies (mAb; 2 mg, single Ab or D10 and B2 1:1). Intra-articular injection was performed under isoflurane anaesthesia. A 1:1 mix of 30 ng CXCL1 and CXCL2 (Sigma) or 30 ng of each was injected into the ankle joint. The CXCR1/2 antagonist reparixin (MedChem Express) was injected subcutaneously twice daily (30 mg/kg/day).

Preparation of human ACPA (hACPA, anti-CCP2 IgG antibodies)

The patients were diagnosed according to the 1987 ACR criteria[16] and determined to be ACPA positive ($ACPA^+$) or ACPA negative ($ACPA^-$) using a routine assay for ACPAs (CCP2 assay). Purification of IgG from plasma and sera from patients with $ACPA^+$ RA, $ACPA^-$ RA and healthy age-matched donors was done as described previously using HiTrap Protein G columns (GE Healthcare).[17] $ACPA^+$ IgG was then further purified using CCP2 affinity column (Euro-Diagnostica). IgG not binding to the CPP2 column was used as control denoted by flow through (FT).

Generation of monoclonal ACPA

Murinised monoclonal D10, B2, C7 and E2 (control) IgG2a were generated as previously described.[18] B cells from the synovial fluid of patients with RA were single sorted, cloned into expression vectors and selected for reactivity for citrullinated epitopes (CEP-1, vimentin and fibrinogen) or control epitope (human tetanus).

Mechanical and thermal hypersensitivity

Withdrawal thresholds of the hind paws were assessed using von Frey filaments as previously described.[19] A 50% withdrawal threshold was calculated using the Dixon up–down method,[20] and the results are presented as per cent of baseline values.

Heat sensitivity was examined using a modified Hargreaves box[21] and cold sensitivity by gently applying a drop of acetone to the hind paw and measuring the duration of nocifensive behaviour (lifting, shaking, biting and licking the paw). The tests were repeated three times on each paw and the average calculated.

Locomotor activity and food/water consumption

Activity level of the mice during a full night cycle was measured using Oxymax/Comprehensive Lab Monitoring System (Columbus Instruments). Mice were monitored during the night cycle (18:00–06:00). Infrared sensors detect movement in X, Y and Z axes and the number of beam breaks recorded and presented as total movement (number of XY-axis beam breaks), ambulation (number of consecutive XY-axis beam breaks) and rearing (number of beam breaks in the Z axis).

Tissue analysis

Metalloprotease activity, western blot, histology, immunohistochemistry, qPCR on tissues and dorsal root ganglion (DRG) functional studies are described in online supplementary methods.

Osteoclast cultures and chemokine analysis

For in vitro osteoclasts generation, bone marrow cells were obtained from wildtype BALB/c mice (Harlan), and $CD\text{-}11b^+$ cells were cultured with M-CSF and RANKL (both Peprotech). Levels of CXCL1 (KC-GRO, Meso Scale Discovery) and CXCL2 (MIP-2α, R&D systems) were measured in the supernatants.

Statistical analysis

For comparing changes over time, repeated measures two-way analysis of variance (ANOVA) was used followed by Bonferroni post hoc test. For differences in three groups or more, one-way ANOVA was used, followed by Bonferroni post hoc test. For differences in two groups, Student's t test was used. Arthritis and histological scores were compared using the Kruskal–Wallis test followed by Dunn's multiple comparison post hoc test. All tests were performed using GraphPad Prism 6 software. p Values <0.05 were considered significant.

RESULTS

Antibodies from human patients with RA induce pain-like behaviour in mice

To examine if RA-associated autoantibodies are directly linked to nociception (pain), we assessed pain-like behaviour in mice after intravenous administration of IgG isolated and pooled from patients with RA or from healthy age-matched donors. A pronounced drop in tactile thresholds was observed in mice injected with 4 mg IgG from patients with $ACPA^+$ RA, but not from patients with $ACPA^-$ RA or healthy individuals (figure 1A).

Pain-like behaviour in mice is only induced by the ACPA fraction of IgG

Three batches (batch 1–3) of affinity-purified ACPA and corresponding FT fractions were prepared, containing ACPA IgG from 38, 6 and 25 patients with $ACPA^+$ RA, respectively. Strikingly, while FT IgG (batch 1, 1 mg) and IgG from healthy individuals (1 mg) injected intravenously did not alter the thresholds for evoking a response to mechanical stimulation, ACPA (batch 1, 1 mg) induced mechanical hypersensitivity within 3 days (figure 1B, C), which lasted for at least 28 days (figure 1C). The ACPA-injected mice displayed increased heat and cold sensitivity compared with saline-injected and FT-injected mice (figure 1E–G). Spontaneous (non-evoked) pain-like behaviour was also assessed. Total movement, ambulation (directional walking) and rearing were monitored in the night between days 2 and 3. Similar assays have been used successfully to study non-reflexive pain-like behaviour in experimental models of pain.[22] Injection of 1 mg purified ACPA (batch 1), but not 1 mg FT IgG (batch 1), induced a reduction in all movement parameters (figure 1H–J). No signs of joint swelling (figure 1D) or inflammation-related sickness behaviour (piloerection, weight loss, reduced feeding, online supplementary figure S1A–C) were observed, thus the reduction in movement is unlikely to be the result of a local or generalised inflammatory event, but rather the consequence of

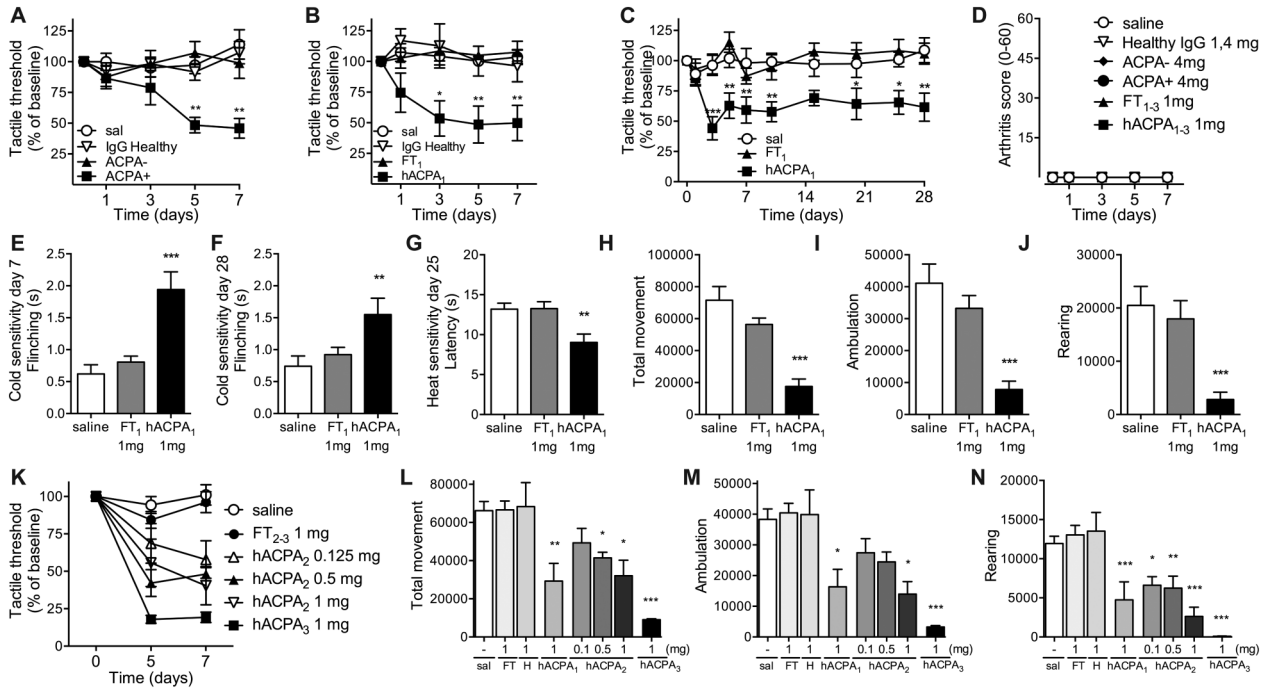


Figure 1 Mechanical and thermal sensitivity and locomotor activity in mice following injection of human antibodies. Mechanical sensitivity in mice injected intravenously with saline (sal), IgG from healthy donors, IgG from patients with ACPA$^-$ RA or IgG from patients with ACPA$^+$ RA (4 mg, n=9/group) (A) and purified human (h) ACPA IgG (batch 1, 1 mg, n=4), non-ACPA IgG from the same patients (FT, 1 mg, n=6) and IgG from healthy donors (1 mg, n=6) (B). ACPA and FT from batch 1 were injected into a different strain of mice (n=7/group) and mechanical sensitivity assessed over time (C), cold sensitivity days 7 and 28 (E and F) and heat sensitivity day 25 (G). Total movement (H), ambulatory (directional) movement (I) and rearing (J) were monitored 12 h the third night (same mice as in C). Arthritis scores (0–60) (D). Mechanical sensitivity days 5 and 7 (K) and total movement (L), ambulatory movement (M) and rearing (N) during third night after injection with 1 mg ACPA batch 1–3 (n=3 each) or 0.5 mg (n=7), 0.125 mg (n=6) ACPA batch 2 or corresponding FT (n=6/group) or saline. Data are presented as mean±SEM. *p<0.05, **p<0.01, and ***p<0.001 are compared with saline. ACPA, anti-citrullinated protein antibodies; FT, flow through; RA, rheumatoid arthritis.

pronociceptive actions of ACPA. Similar results, with a dose-dependent pain-like behaviour, were obtained with the two other batches of affinity-purified ACPA, in two different mouse strains, and also here no effects were seen with FT IgGs (see figure 1K–N and online supplementary figure S1D). These data indicate that the pronociceptive effect of ACPA is neither restricted to a particular batch of ACPAs nor specific for a particular mouse strain.

Monoclonal mouse ACPA induce pain-like behaviour in mice

D10, B2 and C7 murinised IgG2a cloned from single synovial B cells from human patients with RA[18] with varying reactivities for major citrullinated epitopes in RA, but unreactive with the corresponding arginine-containing peptide, were used. Without generating any signs of inflammation (figure 2B), both the murinised D10 and B2 ACPA increased mechanical sensitivity (figure 2C, D) while the C7 ACPA (figure 2C) and E2 (control antibody, figure 2D) did not.

ACPA accumulate in skin, ankle joint and bone marrow

Human ACPA were readily detected in skin, ankle joint, tibial bone marrow and plasma and to some extent in DRG, adipose tissue and spleen, but not in brain or spinal cord (figure 3A) 7 days after injection. Antibodies from healthy controls and FT showed a similar but wider distribution (figure 3A).

ACPA does not induce signs of joint inflammation

Examination of histological sections from ankle joints and tibia 7 days after injection of ACPA, FT and saline, did not display any signs of cell infiltration or synovial hyperplasia (figure 3B–E). No difference in mRNA levels between ACPA-injected and saline-injected mice was observed for the chemokines (*Cxcl5* and *Ccl2*), cytokines (*Tnf*, *Il1b* and *Il6*), inflammatory enzyme (*Cox2*), matrix metalloproteases (*Mmp* 2, 9, 13) and mast cell proteases (*Mcpt4* and *Tpsb2*; figure 3F). Noteworthy, however, *Cxcl1* and *Cxcl2* mRNA levels were elevated in ankle joints from ACPA, but not in FT or saline-injected mice (figure 3F). None of the examined factors were elevated in the skin (see online supplementary figure S2A). ACPA did not induce activation of MMPs in the paws (see figure 3G and online supplementary figure S2B).

ACPA does not increase neuronal excitability in neuronal DRG cultures

To investigate if ACPA have a direct effect on peripheral sensory neurons, we investigated the effects of ACPA on Ca^{2+} fluxes in primary cultures of DRG neurons. Stimulation with FT and ACPA (both 1 μg/mL), followed by KCl (50 mM) to detect cells that can depolarise (ie, neurons) showed an increased intracellular Ca^{2+} signal in 188 cells in response to KCl. Of the KCl responding cells, ACPA and FT stimulation activated six and four cells (2.5% and 1.7%), respectively (figure 4A, B). Thus, the application of ACPA as well as FT had minor effects on Ca^{2+} fluxes, and no difference in response between ACPA and FT was detected.

Electrophysiological recordings in a subpopulation of small diameter nociceptive neurons that express TRPV1 receptors were undertaken using the TRPV1 agonist capsaicin (0.5 μM) at the end of each experiment for verification. A total of 24 cells were patched and recorded in whole-cell voltage clamp mode. Of the 24 cells, 8 cells gave inward current response to capsaicin

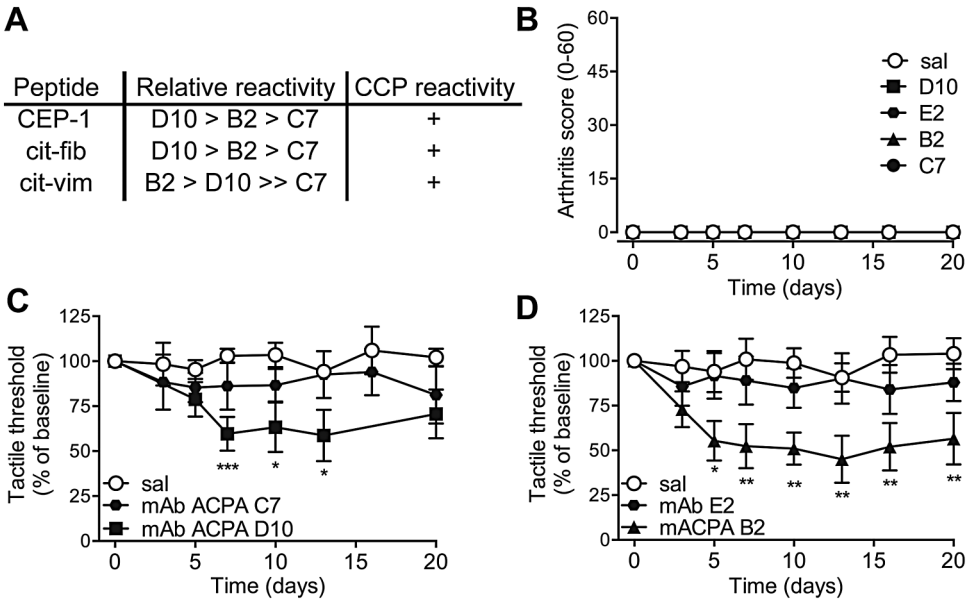


Figure 2 Mechanical sensitivity following injection of murinised monoclonal ACPA in mice. Specificities of the monoclonal antibodies derived from B cells of human patients with RA measured with ELISA,[18] using CEP-1, fib36–52, vim60–75 and CCP peptides. Control antibody E2 binds human tetanus (A). Visual inflammation score (0–60) for all monoclonal antibodies (B). Two milligrams of D10 (n=12) (C), C7 (n=7) (C), B2 (n=7) (D), control antibody E2 (n=7) (D) or saline (sal, n=18) were injected and mechanical sensitivity was measured over 20 days. Data are presented as mean±SEM. *p<0.05; **p<0.01 and ***p<0.001 are compared with saline. ACPA, anti-citrullinated protein antibodies; RA, rheumatoid arthritis.

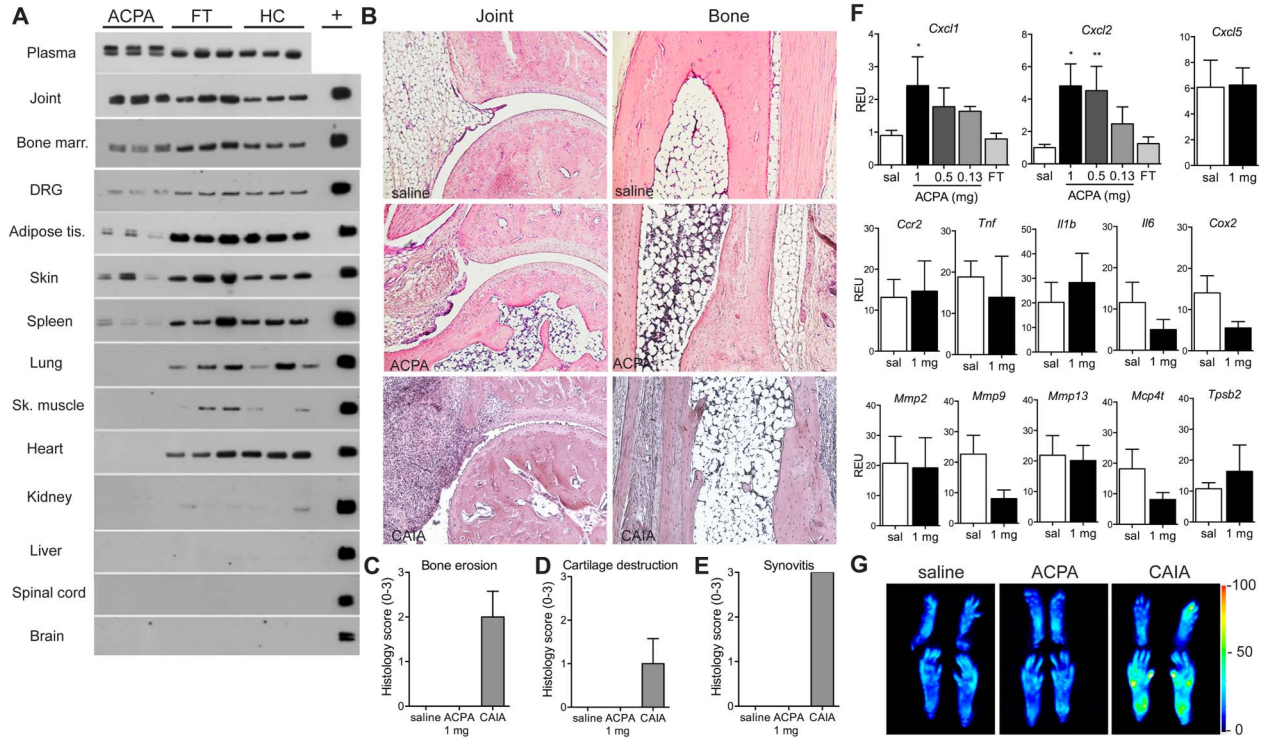
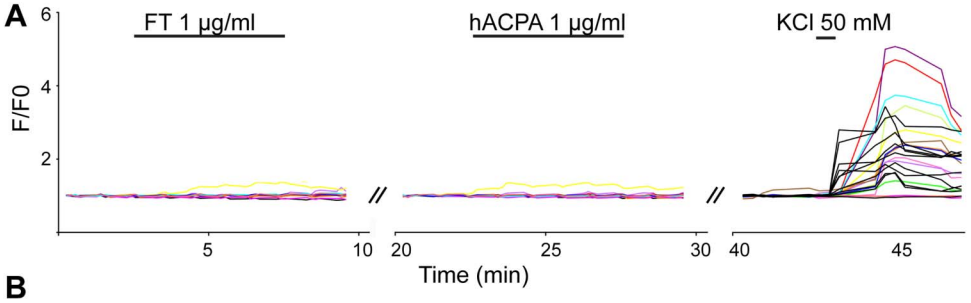
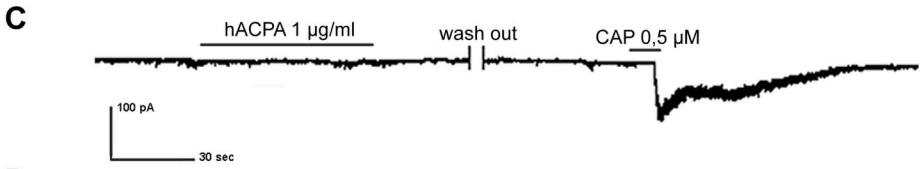


Figure 3 Location of antibodies, histology and gene expression in mice after injection of human ACPA. Mice were perfused with saline (sal) to remove blood, and the presence of human IgG in different tissues 7 days after intravenous injection of 1 mg of $ACPA_3$, FT_3 or IgG from healthy control (HC) was assessed by western blot. Plasma was used as the positive control (A). Representative ankle joint and tibial bone sections stained with H&E 7 days after injection of human $ACPA_3$ (n=3), saline (n=4) or 15 days after induction of collagen antibody-induced arthritis (CAIA, positive control) (n=3) (B) were scored for bone erosion (C), loss of cartilage (D) and synovitis (E). Ankle joint extracts were analysed by qPCR for changes in mRNA levels 7 days after injection of human $ACPA_{2–3}$ (n=6) or saline (n=6) and data expressed as relative expression unit (F). Fluorescence image of paws (G), presented as a heat map after intravenous injection with MMPsense680, which becomes fluorescent in the presence of active MMPs in mice injected with saline, human $ACPA_3$ or anticollagen antibodies as the positive control (n=3/group). Data are presented as mean±SEM; *p<0.05 and **p<0.01 are compared with saline. ACPA, anti-citrullinated protein antibodies; FT, flow through.



B

Total cells	Number of cells responding: ACPA (1 µg/ml)	FT (1 µg/ml)	KCl (50 mM)	% ACPA	% FT	% KCl
234	6	4	188	2,5	1,7	80,3



D

Total cells	Number of cells responding: ACPA (1 µg/ml)	CAP (0,5 µg/M)	% CAP
24	0	8	33

Figure 4 Effect of ACPA on primary peripheral neurons. Mouse dorsal root ganglions were cultured and stimulated with ACPA or FT (both 1 µg/mL). A representative trace showing Ca^{2+} during stimulation with antibodies and KCl (50 mM) (A). Calcium signal were recorded from 243 cells, where few cells showed a minor response to stimulation (2.5% for ACPA and 1.7% for FT) (B). A total of 24 cells were patched and ionic currents were recorded in whole-cell voltage clamp mode (C). None (0/24) of the recorded cells gave inward current response to ACPA, while 33% (8/24) gave response to capsaicin (1 µM) (D). ACPA, anti-citrullinated protein antibodies; FT, flow through.

(33%). No effect of ACPA (1 µg/mL) was seen in any of the investigated cells (0/24 cells, figure 4C, D).

APCA bind to CD68$^+$ cells in vivo and in vitro, and induce CXCL1 release

To determine the cellular targets of ACPA, we performed immunohistochemical labelling of sections from mouse joints and bone. This revealed that ACPA, but not FT control, bind CD68$^+$ cells, which based on CD68 immunoreactivity, multinucleated morphology and proximity to mineralised bone[23 24] most likely are osteoclasts, and cells with the characteristics of osteoclast precursor cells in the bone marrow (see figure 5A and online supplementary figure S3A). ACPA did not label synoviocytes, chondrocytes, osteocytes or PECAM-1$^+$ endothelial cells (see figure 5B and online supplementary figure S3B). Interestingly, some ACPA$^+$ cells were located in very close proximity to CGRP$^+$ sensory fibres in the bone marrow (figure 5C). ACPA immunoreactivity was observed on the cell surface of cultured non-permeabilised CD68$^+$ precursor cells and multinucleated osteoclasts (figure 5D) indicating that the ACPA epitope(s) are expressed on the plasma membrane.

In parallel work in one of our laboratories, we found that interleukin (IL) 8 is released by human osteoclasts in response to ACPA stimulation.[25] We tested if ACPA drive release of IL-8 analogues also from cultured mouse osteoclasts by adding ACPA or FT on day 6, when multinucleated cells (osteoclasts) had started to form. We found that ACPA, but not FT, induced a significant release of CXCL1 (figure 5E) within 4 days in the presence of ACPA, while CXCL2 levels (figure 5F) and number of osteoclasts (figure 5G) did not change.

Pain-like behaviour is dependent on CXCL1/2

Injection of CXCL1 and/or CXCL2 into the ankle joint of mice produced a rapid onset of mechanical sensitivity in the ipsilateral paw, lasting at least 24 h (figure 6A). To examine the functional coupling between ACPA, CXCL1 release and nociception in vivo, mice with mechanical and thermal hypersensitivity induced by intravenous injection of monoclonal ACPA (D10 and B2) were treated with the CXCR1/2 receptor antagonist reparixin. Six consecutive days of reparixin injections (starting day 6) partially reversed mechanical hypersensitivity compared with saline controls between day 16 and the end of the study (day 28) (figure 6B, C) and sensitivity to heat and cold assessed on days 26 and 19, respectively (figure 6D, E). Reparixin treatment or injection of control antibody did not alter tactile thresholds in naive mice (online supplementary figure 4A-B).

DISCUSSION

Here we provide evidence that ACPA, a family of autoantibodies that is common in patients with RA, induces pronounced pain-like behaviour in mice. While we did not identify a direct action of ACPA on sensory neurons, we found that (a) ACPA binds CD68$^+$ osteoclasts in the bone marrow and induces CXCL1/2 expression in the joints and CXCL1 release, (b) intra-articular injection of CXCL1/2 evokes pain-like behaviour and (c) blockade of the chemokine receptors for CXCL1/2 attenuates ACPA-induced hypersensitivity. Thus, our study shows that ACPA-induced nociception is mediated via a mechanism that is dependent on IL-8 release. Importantly, this effect is specific for ACPA, as antibodies isolated from patients who were ACPA$^-$ and

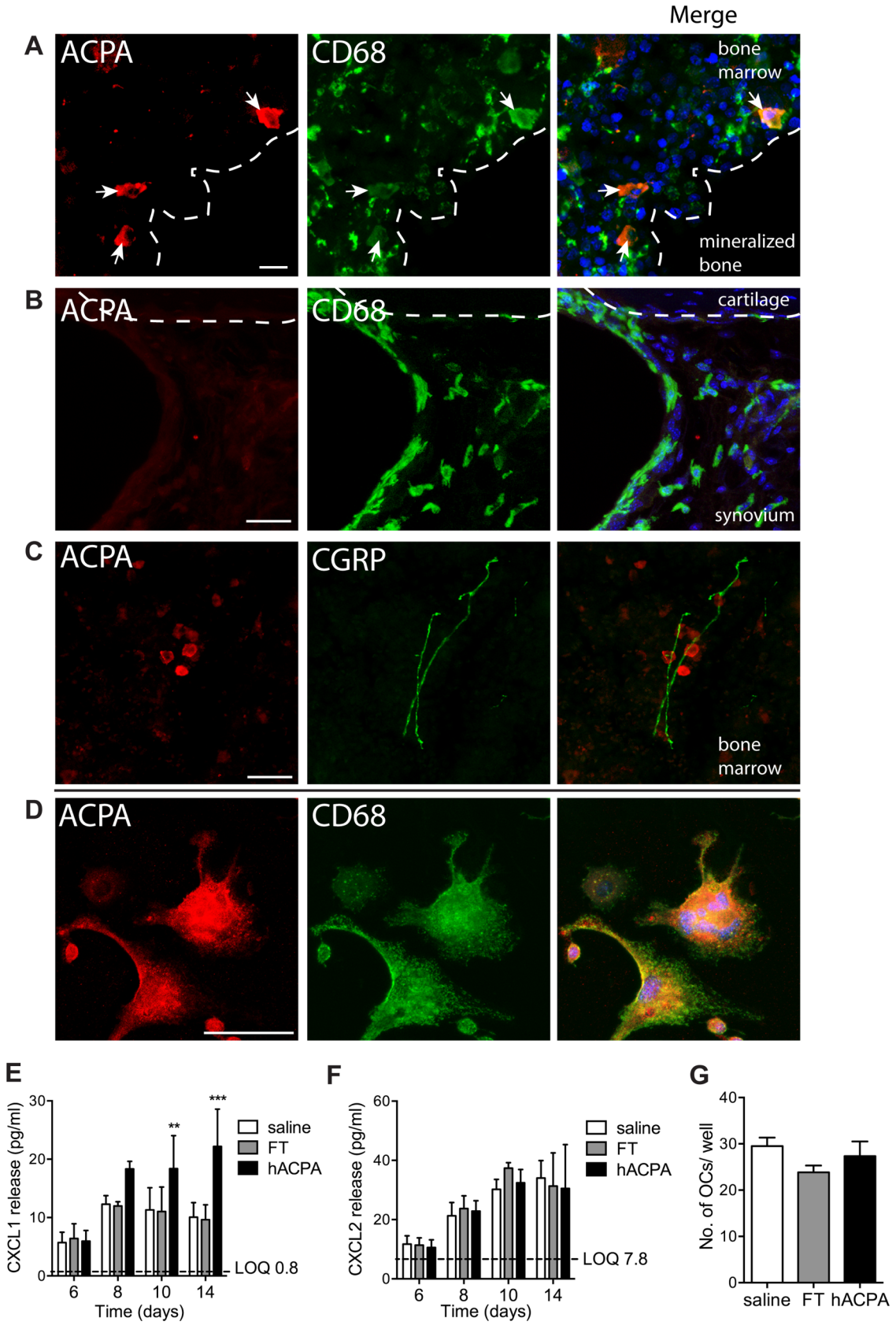


Figure 5 Binding of ACPA in tibial bone marrow and effect of ACPA on cultured osteoclasts. Colocalisation of ACPA: marker for macrophage/osteoclasts (CD68) in subchondral bone (A) and synovia (B), and marker for sensory nerve fibres (CGRP) in tibial bone marrow (C). ACPA and CD68 binding in cultured mouse bone marrow without permeabilisation of the plasma membrane (D). CXCL1 (E) and CXCL2 (F) levels in the supernatant of cultured mouse osteoclasts after stimulation with human ACPA (1 μg/mL), FT (1 μg/mL) or saline (n=6 mice/group). Three different cohorts of littermates were used (E–F). Number of osteoclasts per well at the end of experiment day 14 (G). Data are presented as mean±SEM. **p<0.01 and ***p<0.001 are compared with saline. Scale bar is 25 μm. ACPA, anti-citrullinated protein antibodies; FT, flow through.

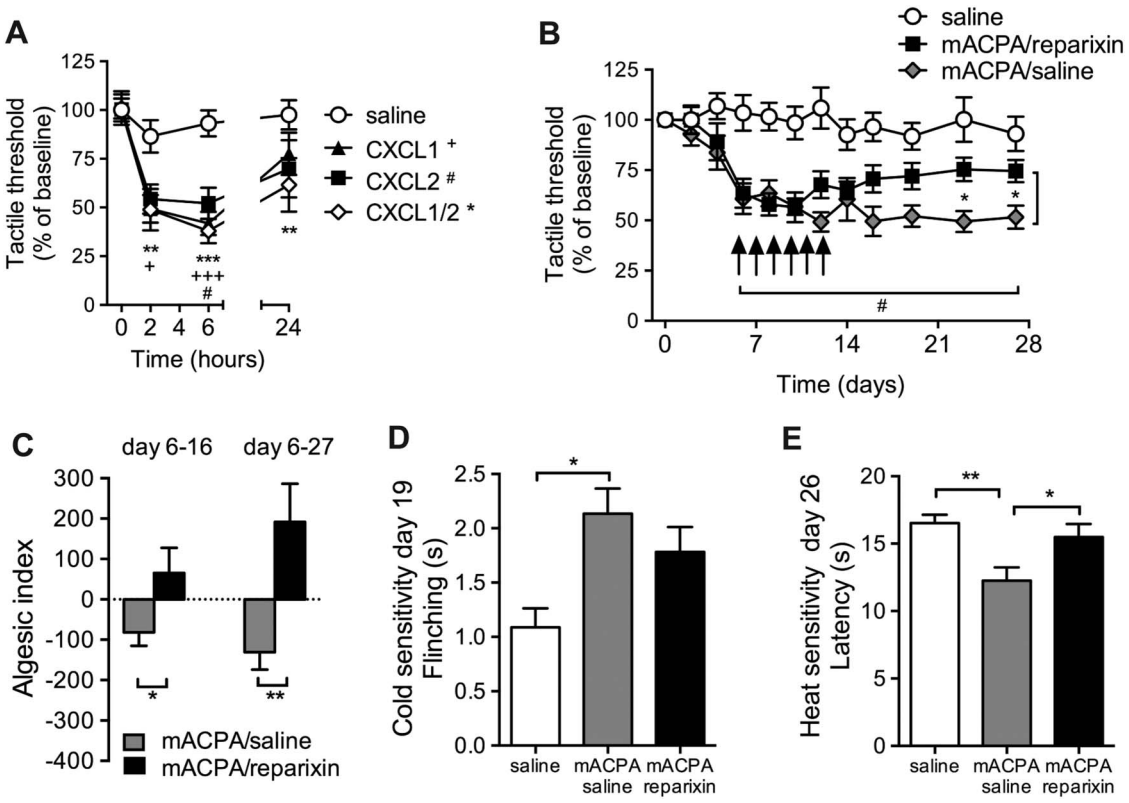


Figure 6 Effect of reparixin on ACPA-induced hypersensitivity. Mechanical hypersensitivity after injection of CXCL1 (30 ng, n=7), CXCL2 (30 ng, n=7) or mixed CXCL1/2 (15 ng each, n=10) or saline (n=20) into the ankle joint (A). Mechanical sensitivity after intravenous injection of mouse monoclonal ACPA D10 and B2 (1 mg each, n=18) or saline (n=9) and treatment with reparixin (30 mg/kg/day, s.c., n=9) or saline (n=9) for 6 days, starting on day 6 (B). Hyperalgesic index comparing area under the curve for reparixin-treated or saline-treated mice from day 6 (C). Cold (D) and heat (E) sensitivity were tested on days 19 and 26, respectively. Results are from two separate experiments. Statistical significance (two-way analysis of variance (ANOVA)) between mACPA/saline and saline is marked by # and difference between mACPA/saline and mACPA/reparixin is marked with * (A). Mechanical sensitivity of mice injected with either saline (n=5) or reparixin (30 mg/kg/day, s.c., n=5) on day 6–12 (E). Data are presented as mean±SEM. * or #p<0.05, **p<0.01 and ***p<0.001 are compared with saline. ACPA, anti-citrullinated protein antibodies.

the FT containing the non-ACPA antibodies from patients who were ACPA$^+$ did not increase mechanical or thermal sensitivity or alter locomotor behaviour in mice. Additionally, murinised monoclonal ACPA induced similar pain-like behaviour in mice as the human ACPA, which excludes potential bias of pronociceptive mechanisms being initiated by immune reactions against human proteins.

We did not observe any visual or histological signs of inflammation in the joints after systemic administration of ACPA. Of the different inflammation-associated factors that were assessed, only CXCL1 and CXCL2 mRNA levels were increased, further strengthening the concept that ACPA-induced pain occurs without the presence of classic signs of inflammation. Thus, our data highlight a potential role of ACPA in the type of joint pain that precedes development of RA and/or persists despite medical control of the disease activity. In the current studies we used polyclonal human antibodies and mouse monoclonal antibodies, which are specific for citrullinated peptides, but which among them have different reactivity patterns for different citrullinated epitopes on different potential target molecules. This variation in reactivity is typical for most of the human B cell and plasma cell derived monoclonals that we have generated so far from RA joints.[18] Since both different batches of polyclonal ACPA and the different monoclonal ACPA varied in their nociceptive potency, it is plausible that certain specific citrullinated epitopes are critical for the induction of pain-like behaviour. This may be part of the reason for why subgroups and not all ACPA$^+$ individuals develop arthralgia. Mapping the nociceptive effect of antibodies with defined fine-specifies will therefore be important in future detailed studies on the molecular mechanisms involved in ACPA-induced pain.

We found that ACPA binds surface epitopes on CD68$^+$ precursor cells and osteoclast in the mouse bone marrow, subchondral bone and growth plate, but did not detect any ACPA-specific immunoreactivity in other regions of the bone and joints, including the synovium. In parallel work,[25] we show that the same monoclonal ACPAs that induced pain-like behaviour also show plasma membrane immunoreactivity in cultured human CD68$^+$ osteoclasts and increase their activity in vitro and reduce bone volume and trabeculations in vivo *in mice* after long-term (4 weeks) exposure to these antibodies. Furthermore, not all ACPA are pronociceptive; the monoclonal ACPA (C7) that lacked pronociceptive properties also failed to induce osteoclast activation in vitro. Thus, there is a correlation between ACPA-mediated effects on osteoclasts and bone metabolism on one hand and activation of the sensory nervous system on the other. Furthermore, while ACPA stimulation evoked IL-8 release from both mouse and human osteoclasts in vitro,[25] ACPA failed to induce inward currents and Ca^{2+} fluxes in cultured mouse DRG neurons, implicating that ACPA does not directly modify neuronal excitability, at least not during the conditions used in the present study. These observations lead to the hypothesis that ACPA induce hypersensitivity via IL-8 release from CD68$^+$ cells in the bone marrow.

The CXCL (IL-8) class of chemokines has been reported to induce pain-like behaviour when injected into peripheral tissues[26 27] or into the spinal fluid[28] of rodents, acting via CXCR2 expressed on peripheral and central nociceptive neurons.[28–30] Furthermore, CXCL1 increases voltage-gated Na^+ and K^+ currents and the function of TRPV1 in murine peripheral DRG neurons, contributing to heightened sensitisation and excitability of the peripheral sensory neurons.[31 32] We detected increased levels of CXCL1 and CXCL2 mRNA in the joints of ACPA-injected mice and injection of CXCL1/2 as a mix, or one by one, into the ankle joint induced mechanical hypersensitivity, confirming a direct involvement of CXCL1/2 in nociception. Since ACPA stimulation did not evoke release of other pain-associated cytokines such as TNF, IL-6 and IL-1β,[25] but caused release of CXCL1/IL-8 in both mouse and human osteoclasts cultures, osteoclasts and CXCL1 provide an intriguing link between ACPA administration and development of pain-like behaviour. We found ACPA immunoreactive cells in the bone marrow located in close proximity to $CGRP^+$ sensory nerves, which provides an intriguing histological link that supports the presence of an interaction between osteoclasts and pain fibres. In addition, blocking the action of CXCL1/2 using reparixin, a CXCL1/2 receptor antagonist in clinical development, reversed ACPA-induced hypersensitivity, pointing to a direct causal relationship between ACPA, CXCL1/2 and pain.

To date citrullinated vimentin[14] and possibly α enolase[25] has been identified as critical epitopes for ACPA-mediated effects on osteoclasts, but other epitopes may also be important. Of note, the Fab part of the ACPA induce osteoclast activation,[25] and thus the reported mechanism is not likely to be solely dependent on Fc-part of the antibodies, although a contribution of activation of Fcγ receptors on osteoclasts cannot be excluded.[33 34] The precise mechanism by which ACPA activate osteoclasts and induce IL-8 release warrants further studies. Our data suggest that CXCL1/IL-8 released from osteoclasts act on nearby sensory neurons through the receptor CXCR2. As we did not observe pain-like behaviour until 2 days after injection of ACPA, it is likely that this process requires ACPA-driven osteoclast differentiation followed by sufficient release of IL-8 to cause peripheral neuronal sensitisation.

In conclusion, our present findings open the possibility that the arthralgia that often precedes the onset of RA may be a direct consequence of the presence of certain ACPA, rather than an unspecific symptom unrelated to the pathogenesis of RA. This insight should dramatically alter our approach to diagnosing as well as treating $ACPA^+$ arthralgia, and may indicate new potential targets for the prevention of development of clinical signs of RA in this early phase of disease development. Such targets would obviously include both IL-8 and associated receptors and factors in osteoclasts that contribute to the ACPA-induced production of IL-8. Our findings may also provide a possible explanation to the remaining pain in some patients with $ACPA^+$ RA who have been successfully treated for their inflammation; levels of ACPA do normally persist also after successful treatment of inflammation.[35 36]

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Correction notice This article has been corrected since it published Online First and in print. The title has been corrected to: Autoantibodies to citrullinated proteins may induce joint pain independent of inflammation. https://ard.bmj.com/content/early/2019/03/20/annrheumdis-2015-208094corr1

Contributors GW and CIS designed experiments, analysed the data and wrote the manuscript along with P-JJ, AIC, KL, RH, VM and LK. GW conducted histology and qPCR, and together with JS, KR, JK and KS behavioural experiments. GW and JMJ-A developed protocols and performed ACPA IHC in joint and bone. DBB and ABF cultured DRG neurons and performed in vitro DRG experiments. CF-C purified human antibodies and performed western blots. AK made osteoclast cultures and associated experiments. AF measured chemokines in supernatants. KSN and RH supplied mice and CAIA antibodies. KA and VM produced mACPA. GW, CIS, DBB, CF-C, JK, P-JJ, AIC, KL, KR, VM, KSN, RH and LK discussed and developed the concept.

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Correction: *Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism*

Wigerblad G, Bas DB, Fernandes-Cerqueira C, *et al*. Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism. *Ann of Rheum Dis* 2016;75:730–. doi:10.1136/annrheumdis-2015-208094.

The specificity of the human monoclonal antibodies B02 and D10 used in functional experiments in this article, originally described as high affinity ACPAs, has been re-evaluated. In accordance with data that were made available to us (Ge *et al*., Arthritis Rheumatol, 2019; 71:210–221, and others), the two monoclonal antibodies used lack specific binding to citrullinated peptides in surface plasmon resonance (SPR) and other assays as described in the retraction note to Journal of Experimental Medicine (Amara et al Retraction J. Exp Med 2019; 216:245). As such the functional results reported for these monoclonal antibodies cannot be attributed to reactivity against citrullinated proteins and/or peptides, but are due to other yet unknown mechanisms. Thus, the pathogenetic implications derived from these experiments cannot be maintained as stated. Specifically, this relates to findings presented in figure 2 A-D and 6 B-E.

Note that in: Figure 2A the table ranking reactivity against citrullinated proteins and/or peptides is based on the original ELISA from the retracted Amara et al Retraction J. Exp Med 2019; 216:245; the results from this ELISA were not reproducible in other assays.

In light of the lack of specificity of these monoclonal antibodies, the functional results observed in Figures 2C-D (reduction in withdrawal thresholds after injection of B02 and D10 monoclonal antibodies); 6B-C (B02/D10-induced mechanical hypersensitivity described as reduction in mechanical hypersensitivity and algesic index); and 6D-E (B02/D10-induced thermal hypersensitivity described as a change in withdrawal latency), confirming the suggested CXCL1 mediation of hypersensitivity using the CXCL1/XCL2 antagonist reparixin, cannot be attributed to reactivity against citrullinated proteins and/or peptides and must have been due to other, hitherto unknown mechanisms.

Since the monoclonal antibodies were used to confirm and expand the data obtained with polyclonal antibody preparations, the remaining conclusions in the paper rely on the data from the polyclonal IgG antibodies purified by affinity chromatography on CCP2-linked Sepharose columns. Although the pronociceptive effects in mice were induced by the CCP2-column eluate IgG (endotoxin free) and not the flow through fractions, also these results have to be interpreted with caution and we conclude that the pathogenetic specificity of these observations and the detailed pronociceptive mechanisms remain to be elucidated. Consequently, we feel that the title on page 730 should be corrected from

"Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism" to: "Autoantibodies to citrullinated proteins may induce joint pain independent of inflammation".

We specifically apologise for the delays from our side in communicating the information in this correction note to the readership of ARD.

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Supplementary data

Supplementary Methods and Materials

Preparation of human ACPAs (hACPA, anti-CCP2 IgG antibodies)

Purification of IgG from humans was done as described previously.[17] Plasma and sera samples (from $ACPA^+$ RA patients, total n = 69, $ACPA^-$ RA patients, n=5 and healthy controls, n=6) were centrifuged at 3000 g for 5 minutes and diluted 1:5 (v/v) in PBS. IgGs were purified from diluted plasma and sera on HiTrap Protein G HP columns (GE Healthcare), according to the manufacturer's instructions. Eluted IgGs were dialyzed against PBS and the antibodies from $ACPA^+$ RA patients were applied to the CCP2 affinity column (kindly provided by Euro-Diagnostica). ACPAs were eluted using 0.1 M glycine–HCl buffer (pH 2.7) and the pH was directly adjusted to 7.4 using 1 M Tris (pH 9). IgG not binding to the CCP2-column were used as control in experiments, denoted as flow through (FT). Autoantibodies were concentrated and the buffer exchanged to PBS using the 10 kDa Microsep™ UF Centrifugal Device (Pall Life Science). Recovery and purity of total ACPAs were analysed by SDS-PAGE followed by Coomassie Blue staining and anti-CCP2 reactivity (Immunoscan CCPlus® assay). The concentration (mg/ml) of total IgG was calculated based on the initial plasma/sera volume applied to the Protein G column and the amount of IgG eluted from the column. The endotoxin levels were determined in the different pools of autoantibodies by the limulus amebocyte lysate assay and the cut-off for positivity was assumed as > 0.05 EU/ml. Three different ACPA pools were utilised for the in vivo experiments: ACPA pool 1 containing autoantibodies purified from 38 plasma samples, ACPA pool 2 containing autoantibodies from 6 plasma/sera samples (plasma n=5; serum n=1), and ACPA pool 3 that includes autoantibodies purified from 25 plasma/sera samples (plasma n=15; sera n=10). To prepare the $ACPA^+$ pool, antibodies isolated from the same plasma/sera samples as used for ACPA pool 2 were selected. This pool of antibodies was constituted by ACPA and non-ACPA IgGs.

Generation of monoclonal ACPA

Murinized monoclonal antibodies (mAbs) D10, B2, C7 and E2 were generated as previously described.[18] In brief, single B-cells were sorted from synovial fluid of ACPA+ patients into a 96-well plate. Digested PCR products from each single cell were cloned into expression

vectors containing Igγ1, Igκ, or Igλ constant regions and transfected into human embryonic fibroblasts HEK293 (Gibco Invitrogen). Supernatants were collected and purified by binding to protein G-sepharose column (Sigma-Aldrich) and expression of heavy and light chain, as well as purity, was verified by PAGE. Reactivity of the generated monoclonal antibodies against citrullinated and native form of α-enolase (CEP-1), vimentin (aa 60-75), and fibrinogen (aa 36-52) peptides was determined with ELISA. The E2 antibody (also derived from a RA synovial B cell) reacts against human tetanus and was detected using ELISA (MyBioSource). Murinization of the human monoclonal antibodies was performed by replacing the full human IgG1 Fc by the murine IgG2a Fc.

Injection of antibodies, CXCLs and CXCR antagonist

Mice were injected intravenously (i.v.) day 0 with either saline or human IgG (hACPA and controls 0.125 -4 mg), mAb ACPA (2 mg, single Ab or D10 and B2 1:1) diluted in 100 µl saline. Intra-articular injection was performed under isoflurane anesthesia. A mix of 15 ng CXCL1 (Sigma) and 15 ng CXCL2 (Sigma), or 30 ng of each was diluted in 3 µl saline and injected into the left ankle (tibio-tarsal) joint. The CXCR2 antagonist reparixin (L-lysin salt, HY-15252, MedChem Express) was injected subcutaneously (s.c. in 100 µl saline) twice daily (30 mg/kg/day).

Mechanical hypersensitivity

Withdrawal thresholds of the hind paws were assessed using von Frey filaments as previously described.[19] In brief, the mice were habituated in individual compartments on top of a wire-mesh surface (Ugo Basile) prior to experiment. On test days, mice were given time to acclimatize and then optiHair filaments (Marstock OptiHair) of increasing buckling force (0.5, 1, 2, 4, 8, 16, and 32 mN) were applied to the plantar surface of the paw until the filament bent slightly. A brisk withdrawal of the paw within 2-3 seconds was noted as a positive response. A 50% withdrawal threshold was calculated using the Dixon up-down method[20] and results from both hind paws were averaged and presented as % of baseline values. After unilateral intra-articular injections only the result from the ipsilateral paw was used. In addition to presenting the results as 50% withdrawal threshold, data (Fig. 6C) were also presented as an algesic index, a calculation that defines the effect of reparixin treatment. It represents the area (based on withdrawal threshold in percent and time in days) between the extrapolated line from start of treatment (day 6) and the time–response curve after reparixin or saline injection. Increasing values indicate decreasing hypersensitivity.

Thermal sensitivity

Heat sensitivity was examined using a modified Hargreaves box.[21] Mice were placed individually in Plexiglas cubicles on the glass surface and allowed to habituate. A radiant heat stimulus was then applied from below to a hind paw until a motion sensor detects a brisk withdrawal and stops the stimulus. Elapsed time is automatically recorded with a cutoff at 20 s. Three measurements from each paw were averaged and presented as latency (in seconds) for the withdrawal.

To assess sensitivity to cold, the mice were placed in the same testing device as used for detection of mechanical hypersensitivity. After habituation a 1 ml syringe was used to gently apply a drop of acetone to the plantar surface of the hind paw and the duration of the nocifensive behavior (lifting, shaking, biting, and licking the paw) was recorded. The test was repeated three times on each paw and the average was calculated.

Locomotor activity and food/water consumption

Food and water consumption, and activity level of the mice during a full night cycle was measured using Oxymax/Comprehensive Lab Monitoring System (CLAMS, Columbus Instruments). Mice were habituated for 24h in single housed testing cages before moved into the CLAMS just before the start of the night cycle (18:00-06:00). Infrared sensors detect movement in X, Y and Z-axes and record amount of beam breaks during the testing period. These values were then accumulated to show total movement over the whole 12 h night cycle. A feeder system connected to a scale and automated water device record consumption during the period. The data is presented as total movement (total number of XY-axis beam breaks), ambulation (number of consecutive XY-axis beam breaks), rearing (number of beam breaks in the Z-axis), food intake (g), and water intake (ml)

Metalloprotease activity

Mice injected with either saline, 1 mg hACPA, or 4 mg anti-CII IgG received i.v. injection of MMPsense 680 (2 nmoles in 150 µl PBS/mouse, PerkinElmer) 24 h before sacrifice. Paws were removed and scanned in an Odyssey CLx (LI-COR) near-infrared system. The signal intensity was quantified and normalized to saline injected mice and the data presented as a heat map.

Tissue analysis

Mice were anesthetized using 4% isoflurane and blood withdrawn by cardiac puncture, followed by saline (with 2 U/ml heparin) perfusion to remove blood before different tissues were dissected, snap frozen and stored in -80°C until further analyses.

Western blot

The presence of human IgG antibodies (ACPA, FT, and IgG from healthy controls) in mouse tissues and plasma was assessed by Western blotting. Joints (ankle), dorsal root ganglia, adipose tissue (subcutaneous white), skin (plantar hind paw), spleen, lung, skeletal muscle (quadriceps), heart, kidney, liver, spinal cord (L4-L6), brain and bone marrow (tibial) were homogenized with protein extraction buffer (0.5% Triton X-100, 50 mM Tris, 150 mM NaCl, 1mM EDTA and 1% SDS, pH 7.4) supplemented with proteases inhibitors (GE Healthcare). Supernatants from the homogenates as well as sera were mixed with LDS sample buffer (Invitrogen) containing DTT, and denatured at 70°C for 10 minutes. Total proteins from the tissues homogenates and plasma (30 µg per well) were loaded onto NuPAGE® Bis-Tris 4-12% gels (Invitrogen) and run in MES-SDS antioxidant-containing running buffer at 200 V for 50 min. Proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) at 30V and blocked with 5% non-fat dry milk prepared in TBS containing 0.1% Tween 20 for 1 hour at room temperature. For the immunoblotting, membranes were incubated with the secondary antibody rabbit anti-human IgG HRP (1:10 000, sc-2769, Santa Cruz Biotechnology) for 1 hour at room temperature. The membranes were developed using the SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific), according to manufacturer's instructions.

Joint histology

Hind ankle joints and tibia from mice injected i.v. with saline, 1 mg hACPA, or arthritis induced with CAIA {Nandakumar:2003hn} were post-fixed in 4% PFA for 48h, decalcified in EDTA (Sigma) for 4-5 weeks, then dehydrated in ethanol and embedded in paraffin. Sections (5 µm) were cut and stained with hematoxylin and eosin (H&E, Histolab) and scored by blinded investigators on a scale from 0-3, where 0 is normal and 3 is severe synovitis, bone erosion, and/or cartilage destruction, as previously described [19].

Preparation of tissue for immunohistochemistry

Naive age-matched mice were deeply anesthetized with pentobarbital (50 μg/kg) and perfused intracardially with saline followed by 4% formaldehyde. Ankle and knee joints were harvested after perfusion and postfixed for 24 hours in the same perfusion fixative. Following this dissection, the joints were decalcified for 3 weeks in 10% ethylenediaminetetracetic acid (EDTA) (PBS, pH 7.4 at 4^{o}C; Sigma); EDTA was changed after 7 days. Following decalcification, each joint was cryoprotected in 30% sucrose at 4^{o}C for at least 48 hours before being sectioned.

To assess the binding of ACPA in different cell popultations, 30 μm-thick frozen sections of the bone/joint were obtained using a cryostat and mouse bone marrow cultures grown on glass slides (detailed below) were used. Then, slides were dried at room temperature (RT) for 30 minutes, washed in 0.01M PBS three times for 10 minutes each, blocked with 3% normal goat serum (NGS, Jackson) in PBS with 0.3% Triton-X 100 (Sigma) for 60 minutes and then incubated overnight with ACPA (5 μg/ml) with different primary antibodies made in 1% NGS and 0.1% Triton-X 100 in 0.01M PBS at RT. Blood vessels were labeled with an antibody against platelet endothelial cell adhesion molecule (rat anti-mouse PECAM, 1:500, BD PharMingen; cat. 550274). Macrophages and osteoclasts were identified with an antibody against a single-chain glycoprotein of 110 kDa that is expressed predominantly on the lysosomal membrane of myeloid cells (rat anti-mouse CD68; 1:2000, cat. MCA1957; AbD Serotec). Multinucleated CD68-positive stained and located within the mineralized bone-lining zone were considered osteoclasts. Primary afferent sensory nerve fibers were labeled with an antibody against calcitonin gene-related peptide (CGRP, polyclonal rabbit anti-rat CGRP; 1:5,000; Sigma; cat. C8198). After primary antibody incubation, slides were washed 3X10 minutes in PBS and incubated for 3 hours at RT with respective (488 and 594) Alexa-conjugated secondary antibodies (1:300; Life Technologies). Sections were washed in 3X10 minutes in PBS and counterstained with DAPI (4', 6-diainimidino-2-phenyl-indole, dihydrochloride, 1:20,000, Molecular Probes) for 5 minutes. After this, preparations were then washed 3X10 minutes each in PBS, dehydrated through an alcohol gradient (459844; Sigma) (70%, 80%, 90%, and 100%) for 2 minutes each wash, cleared in xylene (534053; Sigma) for 2 minutes, and cover slipped with di-n-butylphthalate-polystyrene-xylene (44581; Sigma).

Confocal microscopy

Confocal images were acquired with a LSM710 confocal laser-scanning microscope (Carl Zeiss) operated with ZEN2012 software (Zeiss). Nuclear staining using DAPI; a fluorescent stain that binds strongly to A-T rich regions in DNA was visualized using an excitation beam

of 405 nm and emissions were detected using a BA430-470 emission filter. Sequential acquisition mode was used to reduce bleed-through from fluorophores. Multipanel figures were assembled in Adobe Illustrator CS6 software (Adobe).

Quantitative real-time polymerase chain reaction (PCR)

Ankle joints and plantar paw skin of the hind legs were processed for gene expression analysis. Muscle and tendons were removed from ankle joints, which were then snap frozen and pulverized. Tissues were sonicated in TRIzol (Invitrogen) and RNA was extracted according to manufacturers protocol. After complementary DNA (cDNA) synthesis, quantitative real-time PCR (Applied Biosystems) was performed using hydrolysis probes to determine the relative messenger RNA (mRNA) levels. Primers for chemokines *Ccl2* (MCP1, Mm00441242_m1), *Cxcl1* (Mm04207460_m1), *Cxcl2* (Mm00436450_m1), *Cxcl5* (Mm00436451_g1), inflammatory cytokines *Tnf* (Mm00443258_m1), *Il1b* (Mm00434228_m1), *Il6* (Mm00446190_m1), mast cell proteases *Mcpt4* (Mcp4, Mm00487636-g1), *Tpsb2* (Mcp6, Mm01301240-g1), pro-inflammatory enzyme *Cox2* (Mm00478374_m1), matrix metallo proteases *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. Data is normalized to *Hprt1* values and expressed as relative expression units (REU).

DRG cell culture.

DRGs (L6-C1) from Balb/c mice were extracted and placed in ice-cold Dulbecco`s PBS until enzymatically dissociated with papain (1.7 mg/ml) (30 min at 37°C) followed by a collagenase I (2 mg/ml) and dispase II (8 mg/ml) (Sigma) enzyme mix (30 min at 37°C). The cells were then gently triturated in Leibovitz`s medium supplemented with 10% heat-inactivated bovine serum, 1% penicillin and streptomycin (Invitrogen) and 10 μM mitotic inhibitor (5-fluoro-2-deoxyuridine, Sigma). 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% CO_2 atmosphere and the medium replaced after 24 h and then every third day.

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 $CaCl_2$, 2 mM $MgCl_2$, 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). The 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 15-20 s using a PMT (BioRad MRC 1024). Human ACPA (1 μg/ml) or control FT (1 μg/ml) was applied for 5 min to the same cells with a minimum of 10 min wash period between applications. At the end of each experiment 50 mM KCl was applied for 1 min as a control. All 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 in average 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. F_0 was calculated as the average mean intensity of the first 7 images in each series and the data presented as F/F_0.

Electrophysiological recordings

Whole cell patch-clamp recordings were performed at room temperature (20–22 °C) within 24 and 48 h of culturing using an Axo-Patch-200A amplifier filtered at 1 kHz and sampled at 4 kHz and analyzed 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 $CaCl_2$, 2 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES, 2 mM NaATP, pH 7.15 with Tris-base). Voltage-clamp recordings were conducted in small DRG neurons (15-25 μm in diameter) using the whole-cell configuration. Series resistance was not compensated. DRG neurons were continuously perfused with bath solution (modified HEPES buffer) at a constant flow rate (1-1.5 ml/min). Human ACPA (1 μg/ml) was 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. All reagents were prepared from stock solutions and dissolved in modified HEPES buffer and applied via an 8-channel pressure-controlled application system (AutoMate Scientific).

Osteoclast cultures and Chemokine analysis

For *in vitro* osteoclasts generation, bone marrow cells were obtained from wildtype Balb/c mice (Harlan) and CD-11b^{+} cells were isolated using anti-CD-11b microbeads (Miltenyi Biotec Norden). CD-11b^{+} cells were seeded in 280x10^{5} cells per well in DMEM containing 10% heat inactivated fetal bovine serum (FBS), 2mM L-glutamine, 100 IU/ml penicillin and 50 µg/ml streptomycin (Sigma-Aldrich) and stimulated with M-CSF (Peprotech) 25 ng/ml and RANKL (Peprotech) 25 ng/ml. From day 6, either saline, ACPA, or FT was added to the media (1 µg/ml) purified from peripheral blood of RA patients and medium was replenished every two days with fresh supplements.

Osteoclasts were analysed using Tartrate-resistant acid phosphatase (TRAP) staining by leukocyte acid phosphatase kit 387A (Sigma-Aldrich) following manufacture instructions. TRAP positive cells with not less than 3 nuclei were counted manually as osteoclasts in the Nikon inverted light microscope.

Level of CXCL1 (KC-GRO) and CXCL2 (MIP-2α) was measured in the supernatants from the cultured cells and were analyzed using V-Plex immunoassay kit (Meso Scale Discovery, cat K152QTD-1) for CXCL1 and ELISA kit (R&D systems, cat MM200) for CXCL2, diluted 1:2 in assay diluents and according to manufacturers protocol. Limit of quantification (LOQ) was 0.8 pg/ml for CXCL1 and 7.8 pg/ml for CXCL2.

Statistical analysis

For comparing changes over time, repeated measures two-way analysis of variance (ANOVA) was used followed by Bonferroni post-hoc test. For differences in three groups or more, one-way ANOVA was used, followed by Bonferroni post-hoc test. For differences in 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. All tests were performed using GraphPad Prism 6 software. P values less than 0.05 were considered significant. No statistical method was used to pre determine sample sizes.

Supplemental figures

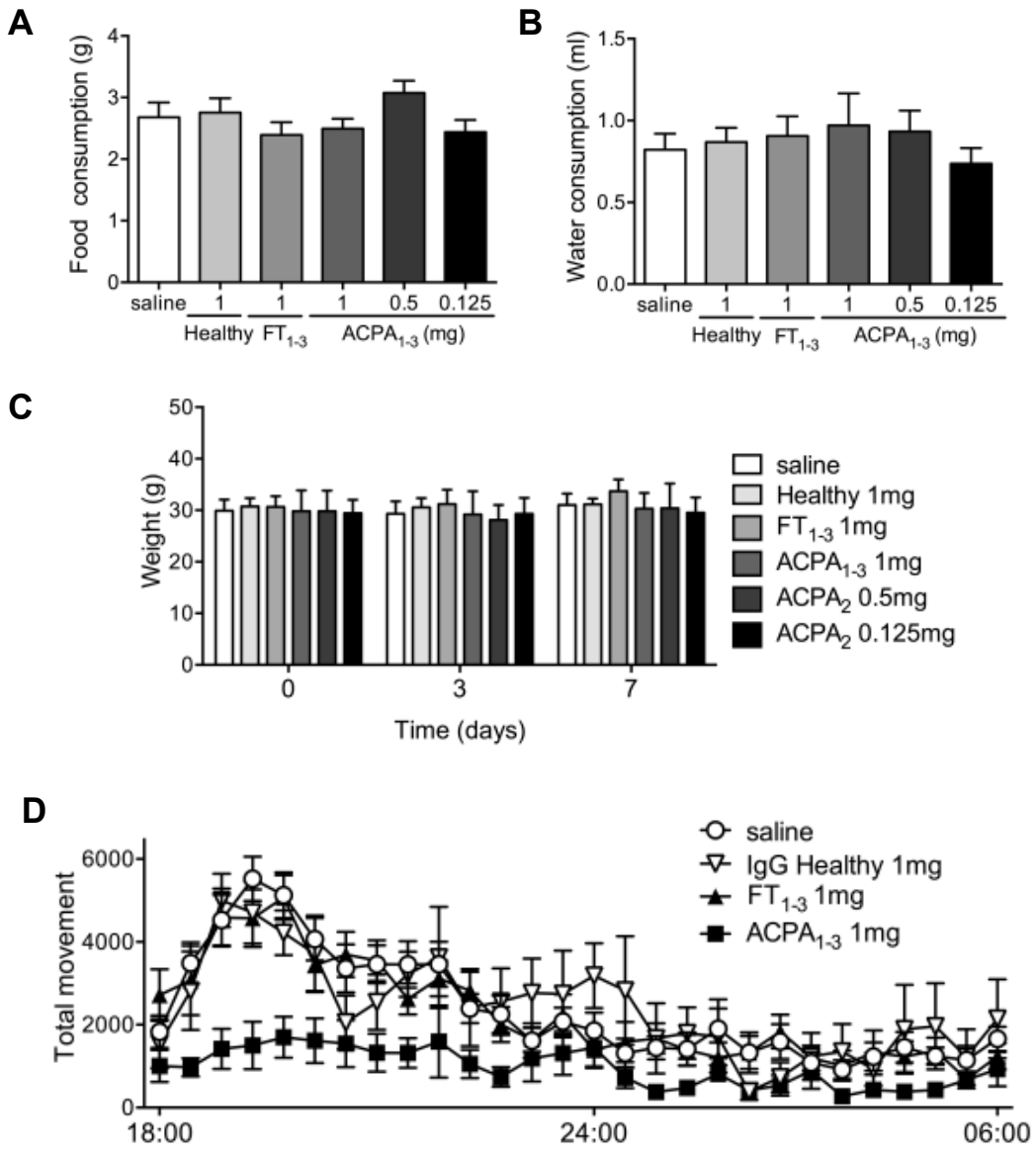
Supplementary figure S1. Locomotor activity and physiological parameters in mice injected with human antibodies. Summary of mice injected with saline (n=18), IgG from healthy donors (n=6), human $ACPA_{1-3}$ (n=10), and FT_{1-3} (n=18), monitored for food (**A**) and water (**B**) consumption, and movement (**D**) during the third night after injection. Body weight of the mice at baseline, 3, and 7 days after injection (**C**).

Supplementary figure S2. Expression of genes in the skin from plantar hind paw in mice and fluorescence after injection of human antibodies. Extracts from the plantar skin of the hind paw were analyzed by qPCR for changes in mRNA levels for different factors 7 days after injection of human $ACPA_3$ or saline and data is expressed as relative expression unit (REU). N.d. means not detectable (**A**). Quantification of fluorescence in the paws after injection of MMPsense, in mice treated injected with $ACPA_3$, saline, or CAIA as positive control (n=3/group). Normalized to saline (**B**).

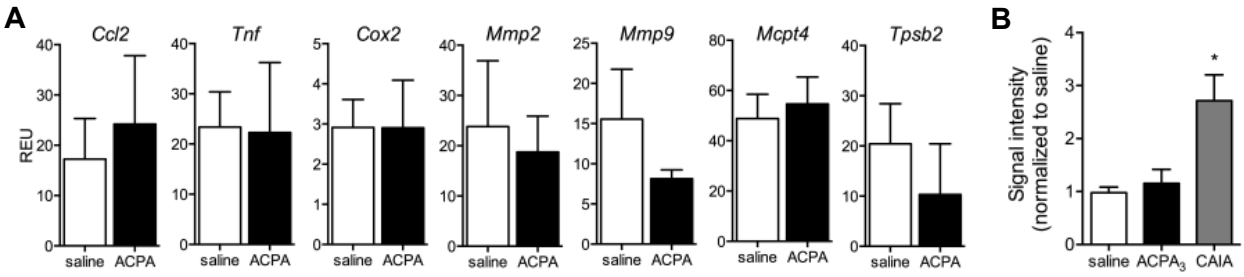
Supplementary figure S3. Binding of ACPA and control antibodies in bone sections. Co-localization of FT and CD68 in subchondral bone marrow of the tibia (**A**). Co-localization of ACPA and marker for endothelial cells (PECAM-1) in the synovia (**B**). Scale bar is 50 µm.

Supplementary figure S4. Effect of reparixin or control antibody on mechanical sensitivity. Mechanical sensitivity of mice injected with either saline (n=5) or reparixin (30 mg/kg/day, s.c. n=5)(**A**). Mechanical sensitivity of mice injected with either saline (n=7) or control antibody G09 (2 mg, i.v. n=8)(**B**).

Suppl. data Fig. 1

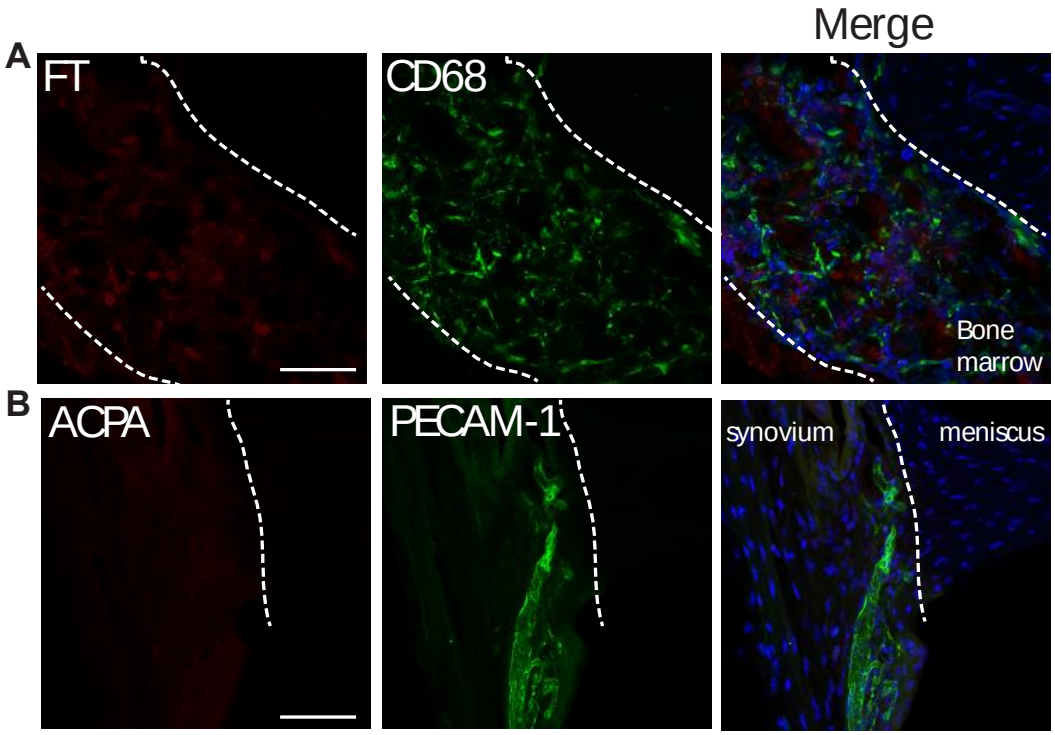


Suppl. data Fig. 2



Cxcl1, Cxcl2, Cxcl5, Il1b, Il6, Mmp13, Mcpt4 n.d.

Suppl. Data Fig. 3



Suppl. Data Fig. 4