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

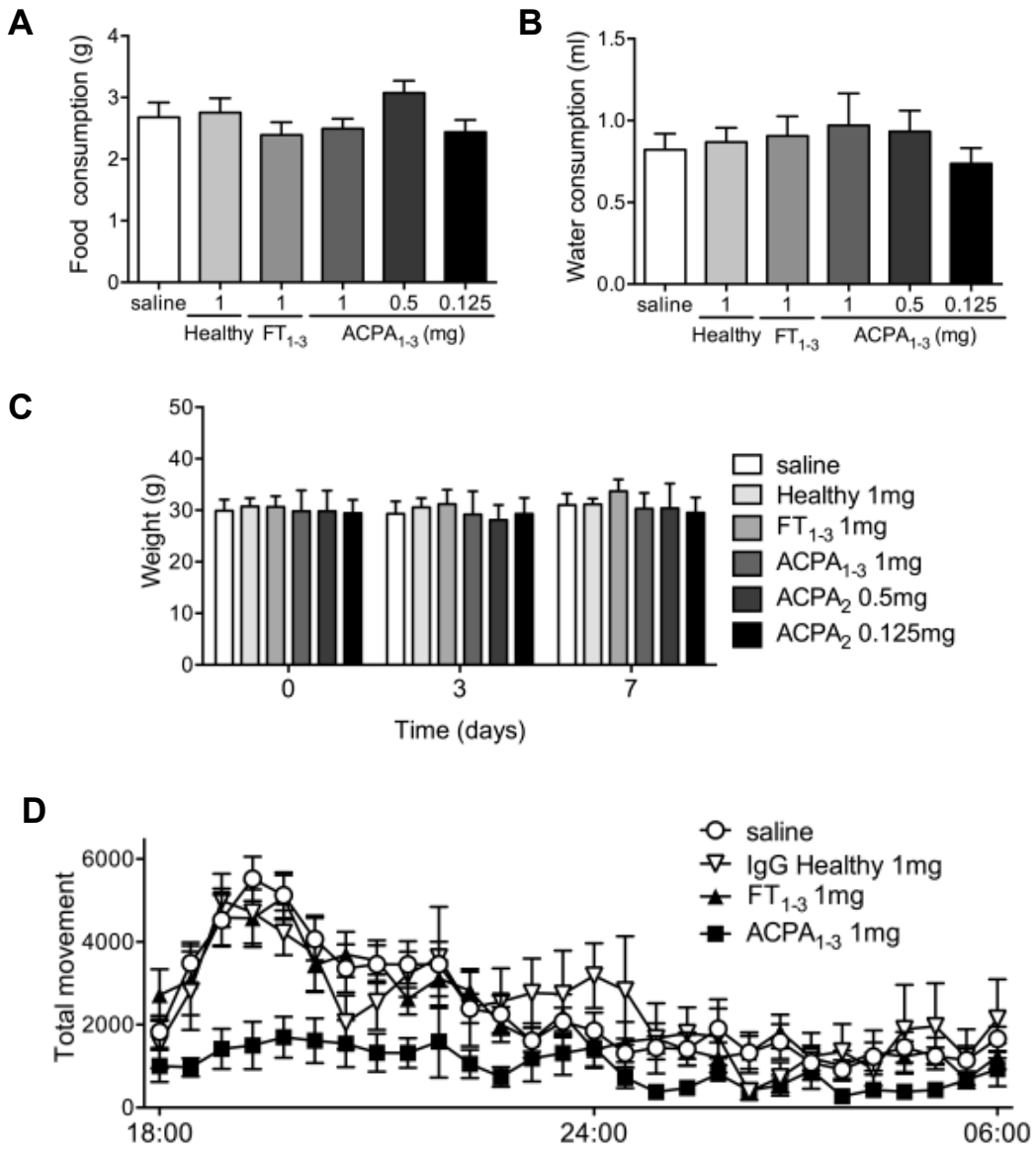
Supplementary figure S1. Locomotor activity and physiological parameters in mice injected with human antibodies. Summary of mice injected with saline (n=18), IgG from healthy donors (n=6), human $ACPA_{1-3}$ (n=10), and FT_{1-3} (n=18), monitored for food (**A**) and water (**B**) consumption, and movement (**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**).

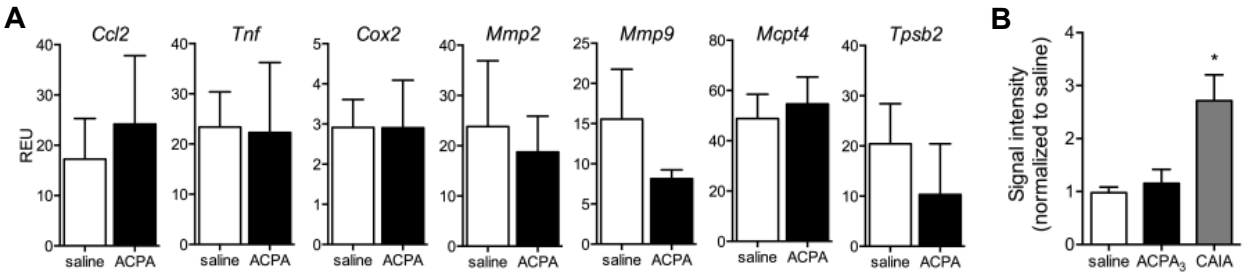
Supplementary figure S3. Binding of ACPA and control antibodies in bone sections. Co-localization of FT and CD68 in subchondral bone marrow of the tibia (**A**). Co-localization of ACPA and marker for endothelial cells (PECAM-1) in the synovia (**B**). Scale bar is 50 µm.

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

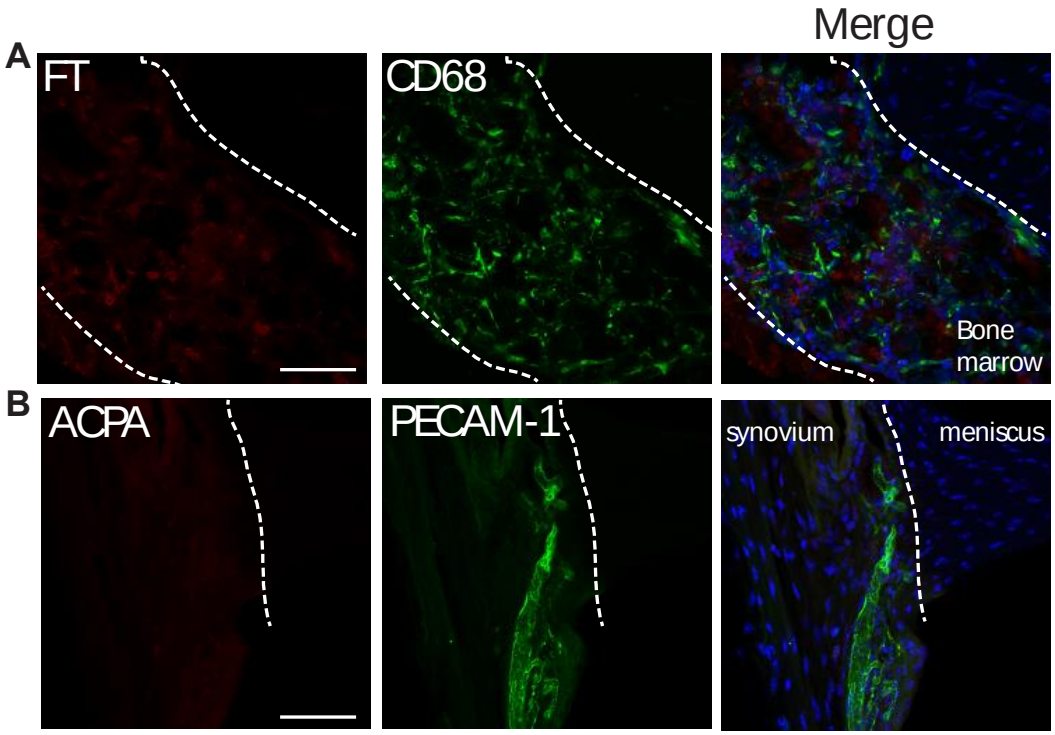


Suppl. data Fig. 2



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

Suppl. Data Fig. 3



Suppl. Data Fig. 4