Online Supplementary Material

Supplementary methods

Proteoglycan content assays. Ethanol-fixed chondrocyte monolayers were stained with Alcian blue to assess proteoglycan content in the extracellular matrix (ECM) *in vitro*. Global proteoglycan content was estimated by the ratio of Alcian blue to crystal violet. In addition, the total amount of proteoglycan released to the culture medium was measured by the 1,9-dimethylmethylene blue colorimetric assay. Frozen sections (5-µm thick) of OCT-embedded cartilage explants were used for Safranin O staining.

Quantitative real-time PCR. Total RNA was extracted from monolayer chondrocytes and quantitative PCR was performed as described previously [1]. Relative mRNA levels were normalized to cDNA level of ribosomal protein L13A (RPL13A) measured within the same plate. Relative expression was calculated by the comparative C_T method ($-\Delta\Delta C_T$) [2]. The gene-specific primers are in supplementary table 1.

Western Blot analysis. For western blot analysis of total cell lysates and culture medium, to detect phosphorylation, monolayers of chondrocytes underwent short-term IL-6 stimulation. Antibodies were obtained for MMP-3 (1:500, ab52915), MMP-13 (1:3000, ab39012), ADAMTS-4 (1:200, ab84792), ADAMTS-5 (1:250, ab41037, all Abcam), actin (1:1000, A2066, Sigma Aldrich), Stat3 (1:1000, 124H6), phospho-Stat3 (1:2000, D3A7), p38 mitogen-activated protein kinase (MAPK) (1:500, 9212), phospho-p38 MAPK (1:500, 4511, D3F9), stress-activated protein kinases (SAPK)/Jun N-terminal kinases (JNK) (1:500, 9252), phospho-SAPK/JNK (1:500, 9251), Akt (1:500, 9272) and phospho-Akt (1:500, 9271, all Cell Signaling), and ERK1/2 (1:100, sc-292838) and phospho-ERK1/2 (1:100, sc-7383, Santa Cruz Biotechnology).

Nitric oxide (NO) and prostaglandin E2 (PGE2) quantification. NO production was measured as the amount of nitrites released into the culture medium, determined by Griess reaction assay [3]. The quantification of PGE2 in culture medium involved a competitive enzyme immunoassay (EIA; PGE2 EIA Kit – Monoclonal, Cayman).

Apoptosis analysis. Chondrocyte apoptosis was determined in monolayer of murine primary chondrocytes by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, using the Apoptag® Apoptosis Detection Kit (Millipore). A counterstain with 4',6-diamidino-2-phenylindole (DAPI) was used to label nuclei, and analyses were performed with an inverted microscope (Axio Observer Z1, Zeiss). The number of apoptotic chondrocytes were quantified in relation with the total number of cells in multiple field images.

Mice. OA was induced in 10-week-old male C57BL/6 mice by DMM of the right knee as described [4]. A mix of ketamine/xylazine and buprenorphin was used to sedate mice, and a second injection of buprenorphin was given the day after surgery [5]. In a first experiment, mice received intraperitoneal injections of a rat anti-mouse IL-6R monoclonal antibody (MR16-1, 0.5 mg once a week, kindly provided by Chugai Pharmaceutical Co.) (n = 10) or phosphate-buffered saline (PBS) as a control (n = 10) for 6 weeks from the day after the DMM. In a second experiment, mice received Stattic (25 mg/kg in 1% Tween 80 PBS every other day) by oral gavage (n = 10) or 1% Tween 80 PBS as a control (n = 8) for 6 weeks from the day after the DMM. Of note, in preliminary experiments, mice received Stattic at a dosage of 50 mg/kg daily. This dosage was associated with severe side effects (weight loss, inertia and death in some animals). Therefore, in subsequent experiments, we used 25 mg/kg every other day, a dosage which did not entail detectable adverse effects. All mice were killed at week 6 and entire knee joints were then dissected and prepared for histology and immunohistochemistry.

Histology. After being fixed in 4% PFA, mouse knee samples were decalcified in 20% EDTA for 10 days and embedded in paraffin. Serial 5-µm-thick sagittal sections of the medial femorotibial joint were collected at 3 depths, at 100-µm intervals. The 3 sections were stained with Safranin O to quantify the OA Research Society International (OARSI) score for cartilage lesions on both tibias and femurs. The total OA score ranged from 0 to 12, and the maximal score for the 3 levels was used for analysis [6]. Assessment of the osteophyte formation, extent of synovitis and bone volume/tissue volume (BV/TV) ratio measurement are described in the online supplementary file. The two readers (AL and EH) were blinded to group assignment.

The extent of synovitis was analyzed on Safranin O-stained sections at the different levels from which the OARSI scores were obtained. The degree of synovitis was scored as previously reported [7]. The thickness of the synovial lining cell layer was evaluated on a scale of 0–3 (0 = 1–2 cells, 1 = 2–4 cells, 2 = 4–9 cells and 3 = 10 or more cells) and cellular density in the synovial stroma on a scale of 0–3 (0 = normal cellularity, 1 = slightly increased cellularity, 2 = moderately increased cellularity and 3 = greatly increased cellularity). Synovitis scores obtained from all four quadrants (medial tibia, medial femur, lateral tibia, and lateral femur) for both of the above parameters were averaged separately and then the sum of averages from both parameters was used for analysis (on a scale of 0–6).

The osteophyte size was assessed with the same Safranin O-stained sections, as previously described [8]: the thickness of the osteophyte was scored on a scale of 0-3 as compared with that of the adjacent articular cartilage (0 = none, 1 = same thickness as the adjacent articular cartilage, 2 = 1-3 times the thickness of the adjacent articular cartilage, 3= more than 3 times the thickness of the adjacent articular cartilage). The mean scores in medial femoral condyle and tibial plateau were then summed.

These sections were also used to study the subchondral bone remodelling. The ratio bone volume/tissue volume (BV/TV) was measured in the subchondral bone (as defined by the region between the calcified cartilage and the growth plate) of the medial side of the tibial

plateau using HistoLab software (Microvision Instruments). The mean BV/TV ratio of the three different sections was computed and used for statistical analysis.

Immunohistochemistry. Immunostaining for phosphorylated Stat3 (pStat3; 1:100) was performed in decalcified paraffin-embedded sections to determine Stat3 activation. Heat-induced antigen retrieval involved incubation with citrate buffer (pH 6.0) at 70°C for 4 hr, followed by a proteolytic method with proteinase K (Sigma). Sections were then blocked with 3% H2O2 for 10 min, and nonspecific binding sites were blocked with 3% normal horse serum and 2% bovine serum albumin for 30 min. Primary rabbit anti-pSTAT-3 antibody was added at 4°C overnight and revealed with ImmPRESS staining reagent (Vector Laboratories) and the diaminobenzidine substrate kit for peroxydase (Vector Laboratories).

To investigate the role of the Stat3 pathway in the effect of IL-6 on cartilage catabolism, cryosections from cartilage explants underwent immunostaining with the antibodies for pStat3 (see above), MMP-3 (1:50), MMP-13 (1:100), ADAMTS-4 (1:600), ADAMTS-5 (1:500, all Abcam), and NITEGE (1:100, Thermo Scientific).

Apoptosis analysis. Chondrocyte apoptosis was determined in monolayer of murine primary chondrocytes by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, using the Apoptag® Apoptosis Detection Kit (Millipore). A counterstain with 4',6-diamidino-2-phenylindole (DAPI) was used to label nuclei, and analyses were performed with an inverted microscope (Axio Observer Z1, Zeiss). The number of apoptotic chondrocytes were quantified in relation with the total number of cells in multiple field images.

Histology. The extent of synovitis was analyzed on Safranin O-stained sections at the different levels from which the OARSI scores were obtained. The degree of

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These sections were also used to study the subchondral bone remodelling. The ratio bone volume/tissue volume (BV/TV) was measured in the subchondral bone (as defined by the region between the calcified cartilage and the growth plate) of the medial side of the tibial plateau using HistoLab software (Microvision Instruments). The mean BV/TV ratio of the three different sections was computed and used for statistical analysis.

Supplementary figures and tables

Supplementary figure S1

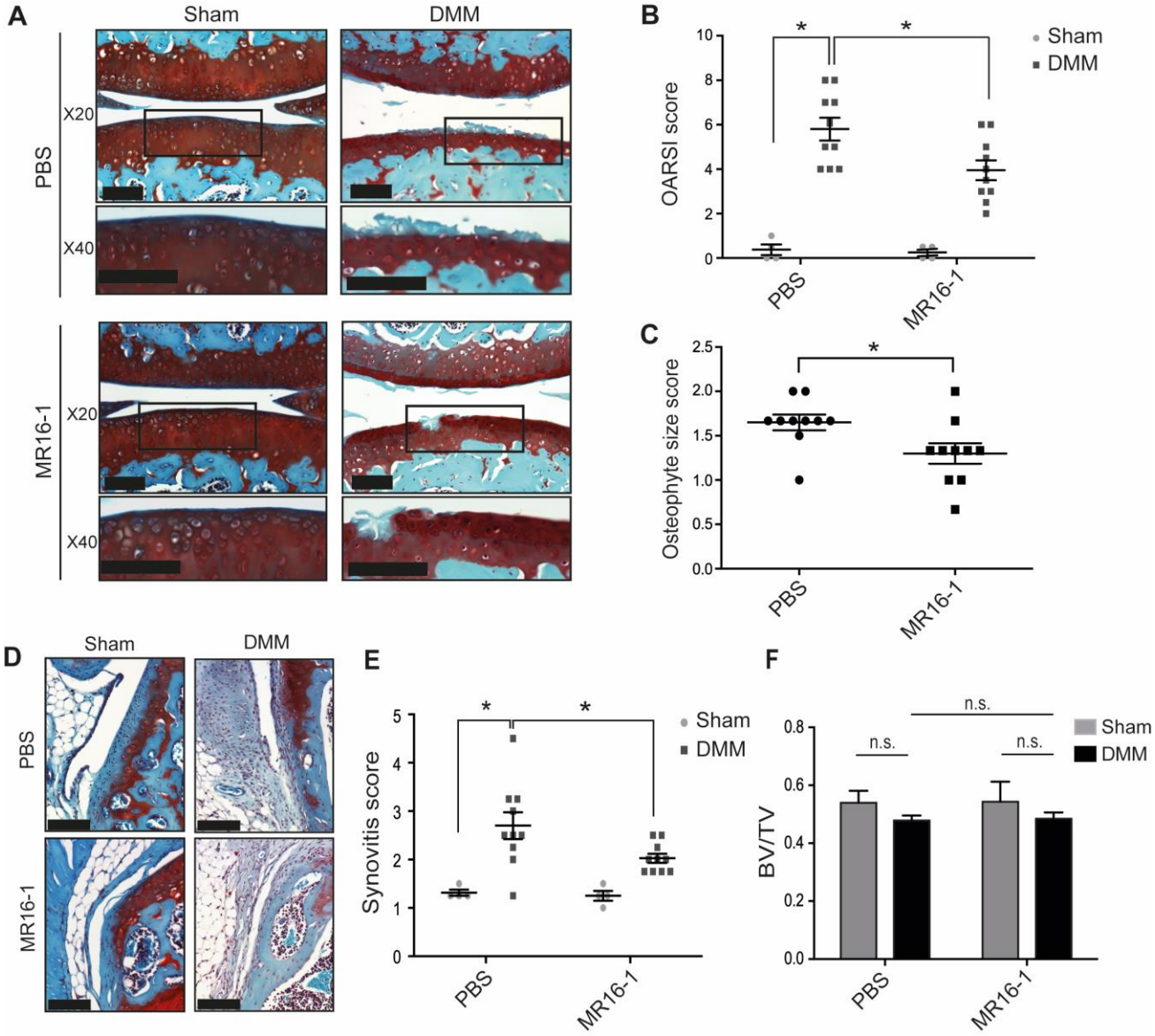

Figure S1. Replication of the experiment investigating the effects of IL-6 systemic blockade in the DMM model of OA, using the right knee from a separate group of mice (n = 4) for sham surgery. MR16-1 was administered for 6 weeks by intraperitoneal injection (0.5 mg) once a week beginning the day after DMM surgery. Phosphate buffered saline (PBS) was used as control (n = 10 mice in each operated group). **A,** Representative Safranin O staining and **B,** OARSI score for medial femorotibial joints of C57BL/6 mice 6 weeks after

destabilization of medial meniscus (DMM) or sham surgery. **C,** Quantification of the osteophyte size in knee joints from mice treated with PBS or MR16-1. **D,** Representative images of the knee synovium and **E,** quantification of the synovial inflammation in knee joints from mice 6 weeks after DMM or sham surgery and treated with PBS or MR16-1. **F,** Histomorphometric analysis of bone volume/tissue volume (BV/TV) ratio in the subchondral bone of the tibial plateau from mice 6 weeks after DMM or sham surgery and treated with PBS or MR16-1. Black scale bar = 100 µm. Data are mean ± SEM. * = $p<0.05$. n.s. = non significant.

Supplementary figure S2

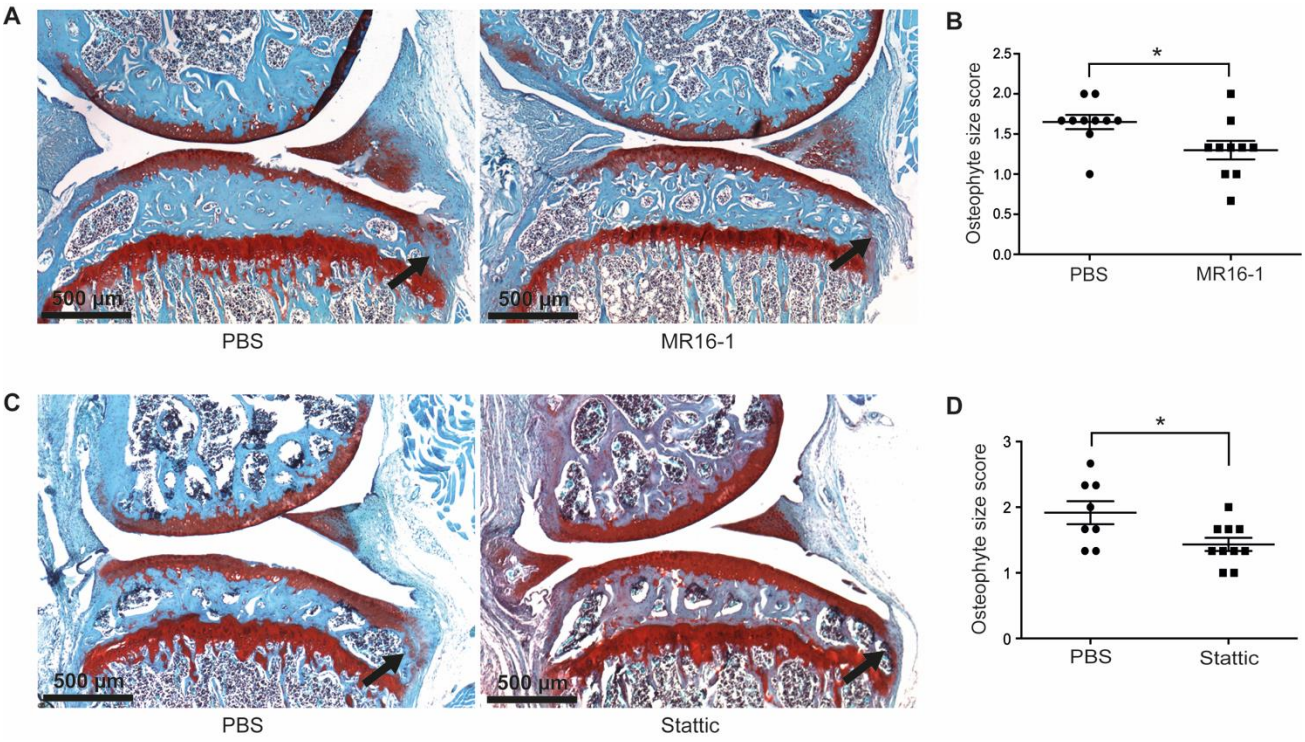

Figure S2. Anti-interleukin 6 receptor (IL-6R) neutralizing antibody (MR16-1) and Stat3 inhibitor (Stattic) both protect against osteophyte formation in experimental osteoarthritis (OA). In a first set of experiments, MR16-1 was injected intraperitoneally (0.5 mg each week for 6 weeks), and phosphate buffered saline (PBS) was used as a control (n= 10 mice in each group). In another set of experiments, Stattic (25 mg/kg every other day beginning the day after DMM) was administered by oral gavage for 6 weeks (n=10) and PBS was a control (n=8). Representative images of Safranin O-stained knee sections from mice subjected to destabilization of medial meniscus (DMM) and treated with MR16-1 **(A)** or Stattic **(C)** for 6 weeks, as compared with their respective PBS control group. The quantification of osteophyte size is shown in the right panel (**B** and **D**, respectively). Black scale bar: 500 µm. Data are mean ± SEM. * = $p<0.05$.

Supplementary figure S3

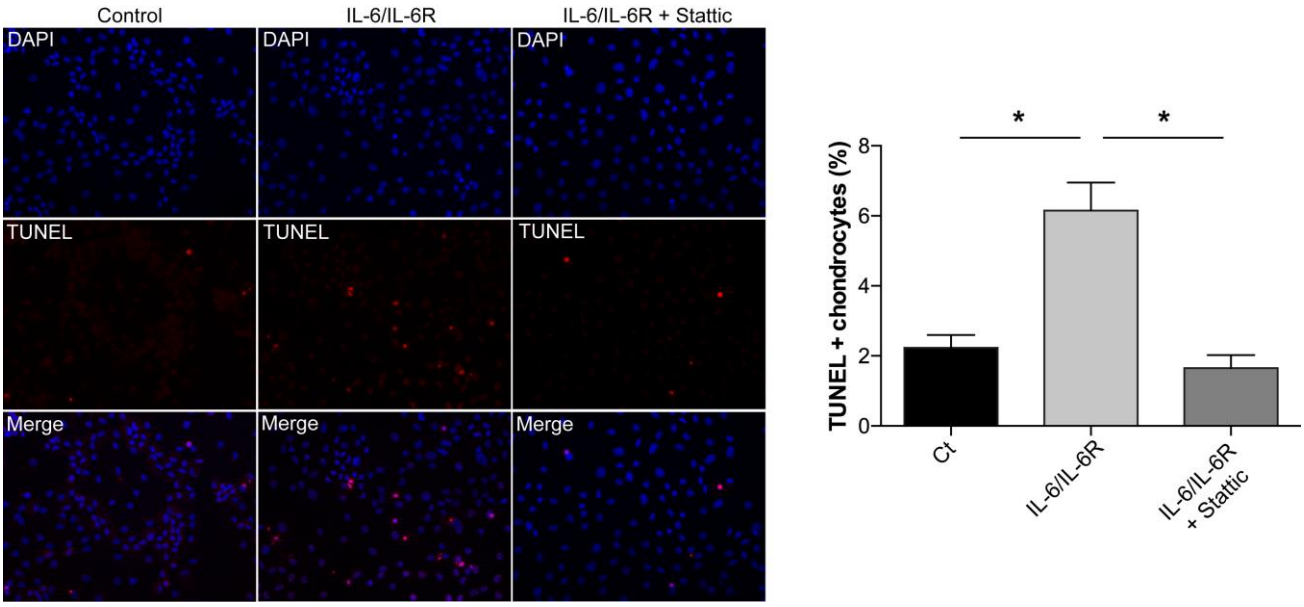

Figure S3. Signal transducer and activator of transcription 3 (Stat3) mediates interleukin 6 (IL-6)-induced chondrocyte apoptosis. Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) staining in monolayers of primary murine chondrocytes treated or not with IL-6 and IL-6 receptor (IL-6R) (100 ng/ml), with or without Stattic (5 µM) for 24 hr (left panel), and quantification of apoptotic chondrocytes in relation with the total number of cells in each condition (right panel). Data are mean ± SEM. * = $p<0.05$.

Supplementary figure S4

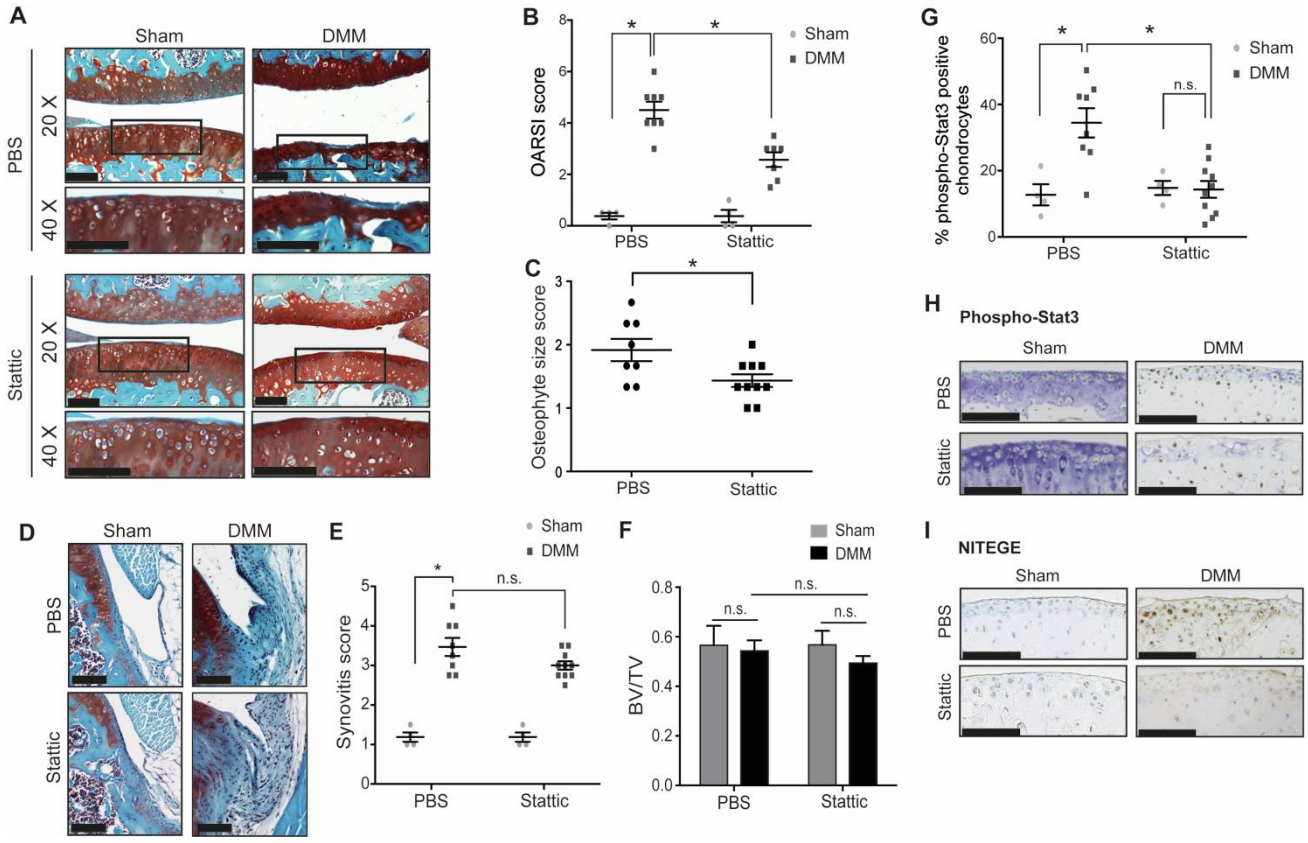

Figure S4. Replication of the experiment investigating the effects of Stat3 systemic blockade in the DMM model of OA, using the right knee of a separate group of mice (n = 4) for sham surgery. Stattic (25 mg/kg) was administered for 6 weeks by oral gavage (n=10 operated mice) every other day beginning the day after DMM surgery. PBS given orally was used as control (n=8 operated mice). **A,** representative Safranin O staining and **B,** OARSI score from medial femorotibial joints of C57BL/6 mice 6 weeks after DMM or sham surgery. **C,** Quantification of the osteophyte size in knee joints from mice undergoing DMM and treated with PBS or Stattic. **D,** Representative images of the knee synovium and **E,** quantification of the synovial inflammation in knee joints from mice 6 weeks after DMM or sham surgery and treated with PBS or Stattic. **F,** Histomorphometric analysis of bone volume/tissue volume (BV/TV) ratio in the subchondral bone of the tibial plateau from mice 6 weeks after DMM or sham surgery and treated with PBS or Stattic. **G,** Percentage of phosphorylated Stat3 (phospho-Stat3)-stained cells in the superficial layer of articular

cartilage in the medial femoral condyle and tibial plateau of Stattic- or PBS-treated mice after DMM or sham surgery. **H**, Representative immunostaining for phosphorylated Stat3 from cartilage of medial femorotibial joints of Stattic- or PBS-treated mice after DMM or sham surgery. **I,** Representative immunostaining of NITEGE in cartilage of medial femorotibial joints of Stattic- or PBS-treated mice after DMM or sham surgery. Black scale bar: 100 µm. Data are mean ± SEM. * = $p<0.05$. n.s. = non significant.

Supplementary figure S5

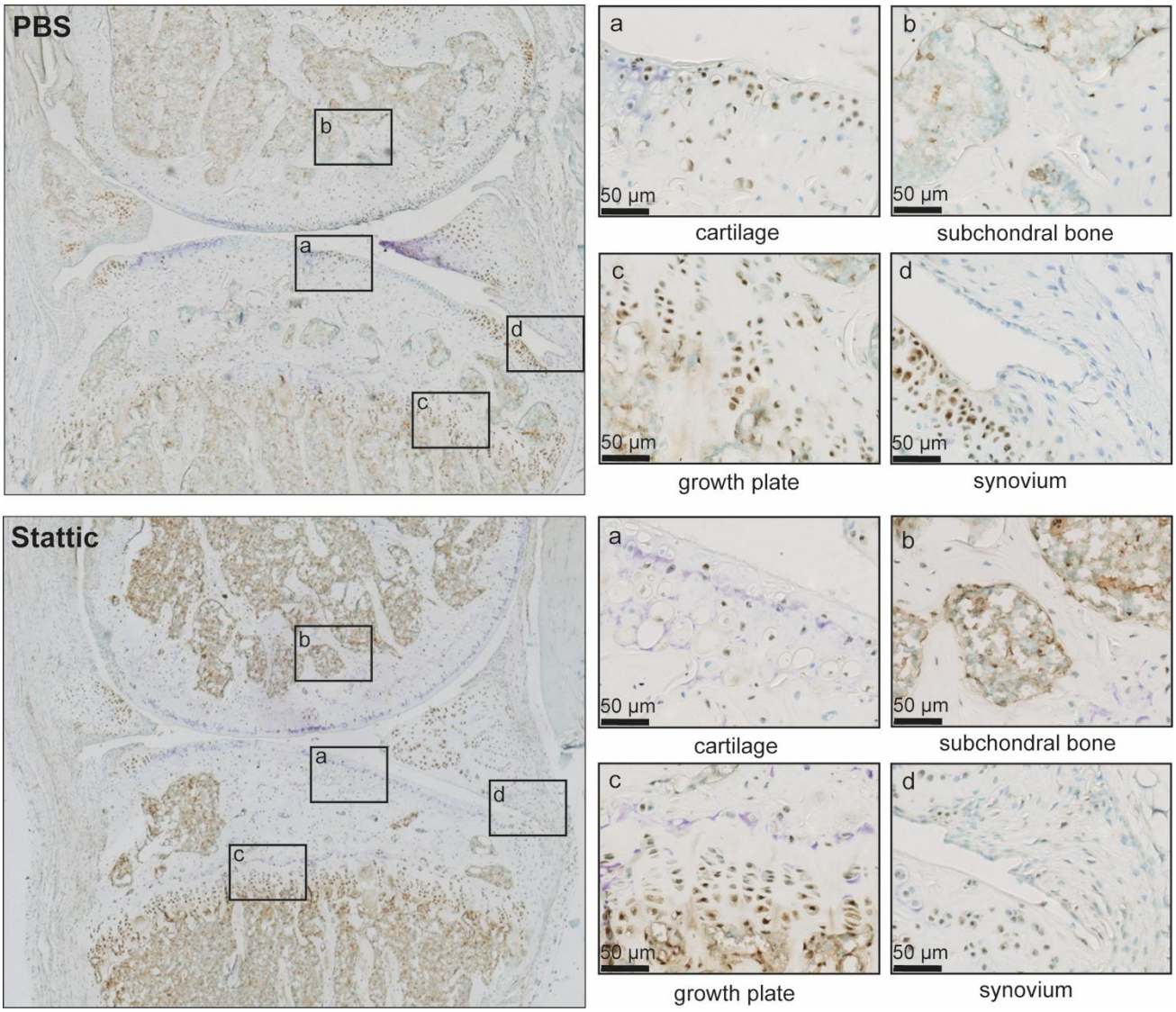

Figure S5. Stat3 activation occurs mostly in articular cartilage in OA mouse knee joints. Representative immunostaining for phosphorylated Stat3 in medial femorotibial joints from mice subjected to destabilization of medial meniscus (DMM) and treated with PBS (upper part) or Stattic (25 mg/kg every other day, lower part) by oral gavage for 6 weeks. Magnifications focusing on articular cartilage (a), subchondral bone (b), growth plate (c), and synovium (d) are shown in the right panel (black scale bar = 50 µm).

Table 1. Gene-specific primers used for quantitative RT-PCR analysis.

Gene	Forward	Reverse
Mmp-3	5'-ATGAAAATGAAGGGTCTTCCGG-3'	5'-GCAGAAGCTCCATACCAGCA-3'
Mmp-13	5'-TGATGGCACTGCTGACATCAT-3'	5'-TGTAGCCTTTGGAACTGCTT-3'
Adamts-4	5'-GGCAAGGACTATGACGC-3'	5'-TCAGCCCAAGGTGAGTG-3'
Adamts-5	5'-TCAGCCACCATCACGAA-3'	5'-CCAGGGCACACCGAGTA-3'
Timp-1	5'-TGGCATCTGGCATCCTCTTG-3'	5'CCATGAATTTAGCCCTTATGACCAG-3'
Timp-3	5'-TCTGCAACTCCGACATCGTG-3'	5'-AAGCCTCGGTACATCTTCATCTG-3'
Coll2	5'-CCGTCATCGAGTACCGATCA-3'	5'-CAGGTCAGGTCAGCCATTCA-3'
Coll10	5'-AAGGAGTGCCTGGACACAAT-3'	5'-GTCGTAATGCTGCTGCCTAT-3'
Aggrecan	5'-CAGGGTTCCCAGTGTTCAGT-3'	5'-CTGCTCCCAGTCTCAACTCC-3'
Vegf	5'-GGAGATCCTTCGAGGAGCACTT-3'	5'-GGCGATTTAGCAGCAGATATAAGAA-3'
Ptgs2	5'-GGTGTGAAGGGAAATAAGGAGC-3'	5'-TCAGGGATGAACTCTCTCCGT-3'
Rpl13a	5'-GGATCCCTCCACCCTATGACA-3'	5'-AGCCGAACAACCTTGAGAGC-3'

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