Supporting Information

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SI Materials and Methods

Patients and Healthy Control Subjects. Synovial tissue specimens were obtained during synovectomy from patients at the Royal Infirmary, Glasgow who had rheumatoid arthritis (RA) or osteoarthritis (OA). Blood and synovial fluid from 12 RA patients and blood from age-matched healthy volunteers ($n = 8$) were used for in vitro studies. The study protocols were approved by the local ethics committees, and all subjects provided signed informed consent.

Animals. Male microRNA species 155-negative (miR155$^{-/-}$) mice on a C57BL/6 (B6) background were purchased from Jackson Laboratories (1). To get WT control littermates, miR-155$^{-/-}$ mice were backcrossed with B6 mice. Both strains then were bred in-house in a pathogen-free facility. Experiments were carried out according to the guidelines of the UK Home Office.

In Situ Hybridization on Paraffin-Embedded Tissues. After deparaffinization, sections were treated with proteinase-K (15 μg/mL) for 10 min at 37 °C. Hybridization with 5 nmol Locked Nucleic Acid 5′ and 3′ digoxigenin (DIG)-labeled scramble (GTGTAACACGTCTATACGCCCA) or miR-155–specific (TATCACGATTAGCATTAA) probes (both from Exiqon) was performed at 50 °C for 1 h. After hybridization, sections were washed in 5× SSC at 50 °C for 5 min, in 1× SSC at 50 °C for two washings of 5 min each, in 0.2× SSC at 50 °C for two washings of 5 min each, and in 0.2× SSC at RT for 5 min. Next, sections were blocked with DIG Wash and Blocking buffer (Roche) in a humidifying chamber for 15 min at RT. Slides then were incubated with alkaline phosphatase-conjugated sheep anti-DIG antibody (1:800; Roche) in blocking solution supplemented with 2% sheep serum for 1 h at RT. Sections were incubated nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolyphosphate (BCIP) solution (Roche) for 2 h at 30 °C. To stop the reaction, slides were washed twice for 5 min with buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 10 mM KCl. Nuclear counterstaining was performed using Nuclear Fast Red (Vector).

Some sections were incubated with fluorescein-conjugated anti-DIG antibody (Roche) followed by incubation with monoclonal mouse anti-human CD68 antibody (DakoCytomation) overnight at 4 °C. On the next day, sections were incubated with biotinylated horse anti-mouse IgG (H+L; Vector) for 30 min at RT followed by incubation with Texas Red Avidin D (Vector) for 30 min at RT. Negative control slides were prepared in the same manner using mouse nonspecific IgG1 (DakoCytomation).

RA and OA tissues were evaluated using a numerical score based on the number of positive cells in the lining, sublining, and perivascular areas of the section (three different fields in each section), with a score of 0 indicating no positive cells; 1 indicating <10% positive cells; 2 indicting 10–50% positive cells; and 3 indicating >50% positive cells.

Induction and Scoring of Experimental Arthritis. ***Collagen-induced arthritis.*** On day 0, mice were injected intradermally with a type II chicken collagen/ Freund's complete adjuvant emulsion (200 μg) (MD BioSciences). On day 21, type II chicken collagen/PBS (200 μg) was injected i.p. Mice were monitored for clinical signs of disease by microcaliper measurements of paw swelling. A clinical severity score was used in which 0 indicated no reaction, 1 indicated swollen digit(s), 2 indicated erythema, 3 indicated a swollen paw/ankle, and 4 indicated loss of function. Mice were killed on day 34.

For histologic assessment, rear paws were fixed for 3 wk in 10% neutral buffered formalin and were decalcified for 3 wk in 10% nitric acid. Paws then were cut into 7-μm sections and stained with H&E.

Collagen antibodies-induced arthritis. Collagen antibodies-induced arthritis (CAIA) was induced in B6 WT or miR-155$^{-/-}$ male mice at 10 wk of age according to a previously described method (2). Briefly, on day 0 mice received the anti-collagen II antibody mixture (8 mg per mouse i.v.) (MD Biosciences). On day 3, 100 μg of *Escherichia coli* LPS (055:B5; Sigma) was administered i.p. Mice were monitored for clinical signs of disease using microcaliper (Kroeplin GmbH) measurements and a clinical score based upon disease severity (0 = no reaction; 1 = mild but definite redness and swelling of the ankle/wrist/digits; 2 = moderate-to-severe redness and swelling of the ankle/wrist; 3 = redness and swelling of the entire paw including digits; 4 = maximally inflamed limb with involvement of multiple joints). Mice were killed on day 14.

Serum and Cell Supernatant Analysis. Levels of IgG2a and IgG1 anti-collagen antibodies in sera were measured using enzyme-linked immunoassay. Briefly, 96-well plates were coated with type II chicken collagen in PBS, pH 7.4 (0.4 μg per well). Serum samples (100 μL) serially diluted in PBS were incubated for 1 h, followed by the addition of biotin anti-mouse IgG2a or IgG1 (BD) and then ExtrAvidin peroxidase (Sigma). Each 1-h incubation step was followed by four wash/rinse cycles of immersion in PBS containing 0.5% Tween 20. Peroxidase substrate was added, and color development proportional to antibody activity was measured by optical density read at 450 nm. Cytokine levels in serum samples or cell culture supernatants were measured using a 20-plex or 30-plex cytokine assay or paired antibodies by ELISA (Invitrogen).

Cell Culture. To establish the transfection efficiency, CD14^{+} cells were transfected with miR mimic labeled with Dy547 using the N-TER nanoparticle siRNA transfection system (Sigma). After 48 h cells were collected and analyzed by flow cytometry (FL-2). To determine at the antigen-specific T-lymphocyte response, draining (popliteal) lymph nodes cells from WT and miR-155$^{-/-}$ mice that underwent the CIA protocol were analyzed as described previously (3). Briefly, cells were stimulated with phorbol 12-myristate 13-acetate/ionomycin for 4 h; GolgiStop was added during the final 3 h. The cells then were incubated with anti-mouse CD16/32 antibodies (BD Biosciences) to minimize non-specific Fc-staining, followed by allophycocyanin (APC)- or phycoerythrin (PE)-conjugated anti-CD4 (BD Biosciences) and PE- or APC-conjugated anti–IL-17, anti–IL-4, or anti–IFN-γ antibodies as appropriate (all from BD Biosciences).

Quantitative PCR. Total RNA was isolated using the miRNeasy kit (Qiagen). The miScript Reverse Transcription Kit (Qiagen) was used for cDNA preparation. TaqMan mRNA assays (Applied Biosystems) or miScript primer assay (Qiagen) were used for semiquantitative determination of the expression of mouse TNF-α (Mm00443258_m1*), mouse IL-6 (Mm00446190_m1*), mouse IFNγ (Mm01168134_m1*), human Src homology 2-containing inositol phosphatase-1 (SHIP-1) (Hs00183290_m1), mouse SHIP-1 (Mm00494663_m1), mouse miR-155 (MS00001701), and human miR-155 (MS00003605). The expression of U6B snRNA or β-actin was used as endogenous control.

Luciferase Activity Assay. The human SHIP1 miRNA target site was generated by annealing the oligos:

SHIP1 3′ UTR forward; CTTGCACTGGGCTTCTTAATG-CTTTCACCCCTCCGAACACACACCGTTTGGATCCA
SHIP1 3′ UTR reverse: TAGTGGATCCTAATTGTGCAG-GTACAGGAATTGTTCCACCAGCATTAGGAACTTT-AGCATA

which were cloned in both sense and antisense orientations downstream of the luciferase gene in pMIR-REPORT luciferase vector (Ambion). These constructs, named "pMIR-SHIP1-miR155" and "pMIR(A/S)-SHIP1-miR155," were sequenced to confirm inserts and were used for transfection of HEK293 cells. HEK293 cells were cultured in 96-well plates and transfected with 0.1 μg of pMIR-SHIP1-miR155, pMIR(A/S)-SHIP1-miR155, or pMIR-REPORT, together with 0.01 μg of pRL-TK vector (Promega) containing *Renilla* luciferase and 40 nM of miR-155 or scrambled miRNA (Thermo Scientific Dharmacon). Transfections were done using Effectene (Qiagen) according to the manufacturer's instructions. Luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega) 24 h after transfection.

Human SHIP-1 Staining. Immunofluorescence was performed on formalin-fixed RA and synovial tissues with a double immunofluorescence procedure using a primary antibody against CD3 (mouse monoclonal anti-human CD3, VP-C316; Vector Laboratories), anti-CD68 (mouse monoclonal anti-human CD68, M0876; DakoCytomation), anti-CD20 (mouse monoclonal anti-human CD20, M0755; DakoCytomation) or anti–SHIP-1 (mouse monoclonal anti-human SHIP-1, SC8425; Santa Cruz Biotechnology). Sections then were rinsed and incubated with the appropriate secondary biotinylated antibodies (biotinylated horse anti-mouse IgG, BA-2000; Vector) and Avidin D fluorochrome conjugates (fluorescein, A-2001, or Dy-Light-594, SA-5594; Vector). Briefly, 5-μm sections of paraffin-embedded tissue were deparaffinized in xylene and rehydrated through graded alcohols to water. To block endogenous peroxidization, sections were incubated with 0.5% H_2O_2/methanol. Next, epitope retrieval was performed in citrate buffer, pH 6.0. Sections then were incubated with 5% horse serum and primary antibodies followed by incubation with secondary biotinylated antibodies and Avidin D fluorochrome conjugates. Slides were mounted in medium containing DAPI (H-1200; Vector) and were scanned on a Zeiss Axiovert S 100 fluorescent microscope.

TaqMan Low-Density Arrays. TaqMan low-density arrays (TLDA) are 384-well microfluidic cards that allow multiplex PCR. Primer sets for a range of inflammatory cytokines and chemokines were generated and used to assess transcriptional fold changes using 18S as an internal control. Macrophages generated from murine miR-155$^{-/-}$ and WT bone marrow precursors were treated with PBS or ultrapure LPS (100 ng/mL) (Invivogen Ltd) for 6 h. Macrophage cDNA (1 μg per sample) was generated via the Qiagen RNeasy and Affinityscript cDNA kits, then was loaded onto TLDA plates after 1:1 dilution with TaqMan PCR Master Mix No AmpErase (ABI Ltd), and was run on a 7900HT TaqMan reader using SDS software. ΔCt values were generated via the 18S control, and ΔΔCt values were obtained by normalizing the mean ΔCt with the untreated control. Relative quantitative values were calculated as 2^-ΔΔCt. Results are expressed as fold change over untreated control.

Statistical Analysis. Statistics were performed using the Mann–Whitney test or ANOVA followed by Tukey's test or student's *t*-test using SPSS Software. All data are expressed as mean ± SEM. $P < 0.05$ was considered significant.

1. Thai TH, et al. (2007) Regulation of the germinal center response by microRNA-155. *Science* 316:604–608.
2. Nandakumar KS, Holmdahl R (2007) Collagen antibody induced arthritis. *Methods Mol Med* 136:215–223.
3. Xu D, et al. (2008) IL-33 exacerbates antigen-induced arthritis by activating mast cells. *Proc Natl Acad Sci USA* 105:10913–10918.

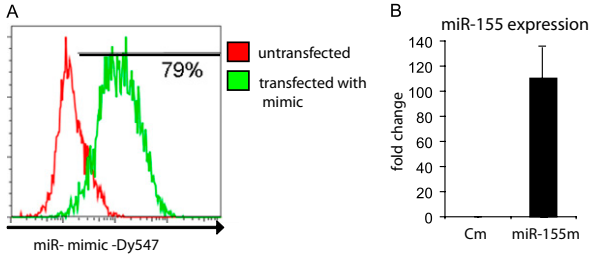

Fig. S1. Efficient transfection of human CD14$^+$ cells with microRNA mimic. Peripheral blood CD14$^+$ cells were transfected with miR-155 mimic or control scramble mimic labeled with Dy547 (both 20 nM) using the N-TER nanoparticle siRNA transfection system or were left untransfected. After 48 h, cells were analyzed by flow cytometry, or RNA was isolated and quantitative PCR for miR-155 and endogenous control snU6B was performed. (*A*) Seventy-nine percent of CD14$^+$ cells were considered transfected with labeled mimic. (*B*) Expression of miR-155 normalized to snU6B in cells transfected with miR-155 and scramble mimic is shown. Cm, control scramble mimic; miR-155m, miR-155 mimic. Data are means ± SEM of three experiments. *$P < 0.05$, control vs. miR-155 mimic.

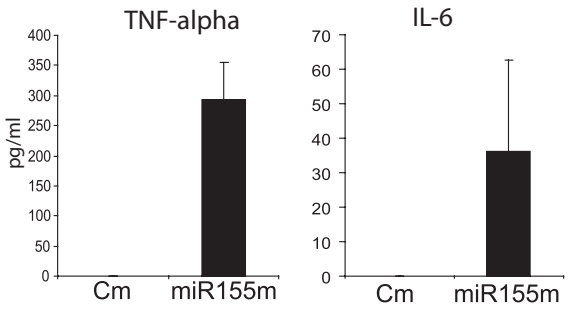

Fig. S2. miR-155 promotes spontaneous production of proinflammatory cytokines by human macrophages. $CD14^{+}$-derived macrophage colony-stimulating factor differentiated macrophages were transfected with miR-155 mimic (miR-155m) or control scramble mimic (Cm) (both 20 nM) using Dharma FECT 3 transfection reagent (Thermo Scientific). Cell supernatants were analyzed 48 h later for IL-6 and TNF-α. Data are means ± SEM of two experiments.

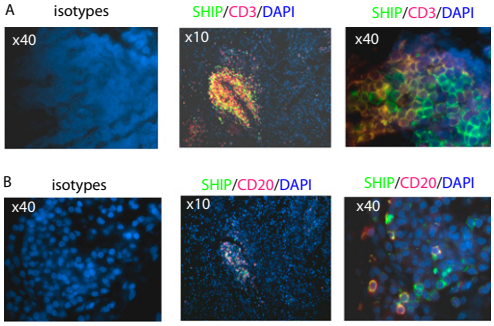

Fig. S3. SHIP-1 is expressed in both T and B cells in RA synovium. (*A* and *B*) Synovial specimens from RA (n = 5) and OA (n = 5) patients were incubated with anti-human SHIP-1 and either anti-human CD3 (*A*) or anti-human CD20 antibody (*B*), or appropriate isotype controls. Representative staining from one of five specimens with similar results is shown; blue, DAPI staining; red, CD3+ or CD20+; green, SHIP-1+; yellow, SHIP-1+/CD3+ or SHIP-1+/CD20+ cells. Magnification is as indicated.

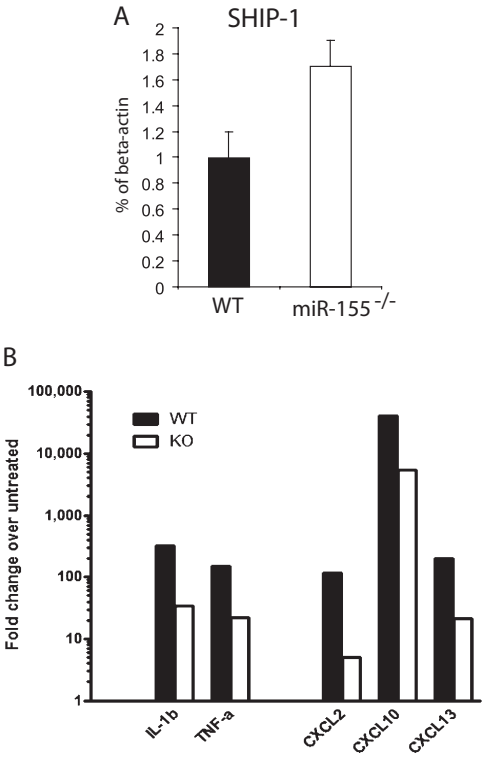

Fig. S4. The expression of SHIP-1 and proinflammatory cytokines/chemokines in miR-155$^{-/-}$ macrophages. (*A*) SHIP-1 is up-regulated in miR-155$^{-/-}$ macrophages. RNA from WT and miR-155$^{-/-}$ bone marrow-derived macrophages stimulated with LPS (100 ng/mL) for 24 h was isolated, and expression of mouse SHIP-1 was measured with quantitative PCR. Data are means ± SEM of two independent experiments. (*B*) miR-155$^{-/-}$ macrophages show lower expression of proinflammatory cytokines/chemokines. WT and miR-155$^{-/-}$ bone marrow-derived macrophages were incubated with medium or LPS (100 ng/mL). After 6 h, total RNA was isolated, and mouse cytokine/chemokine TLDA was performed.

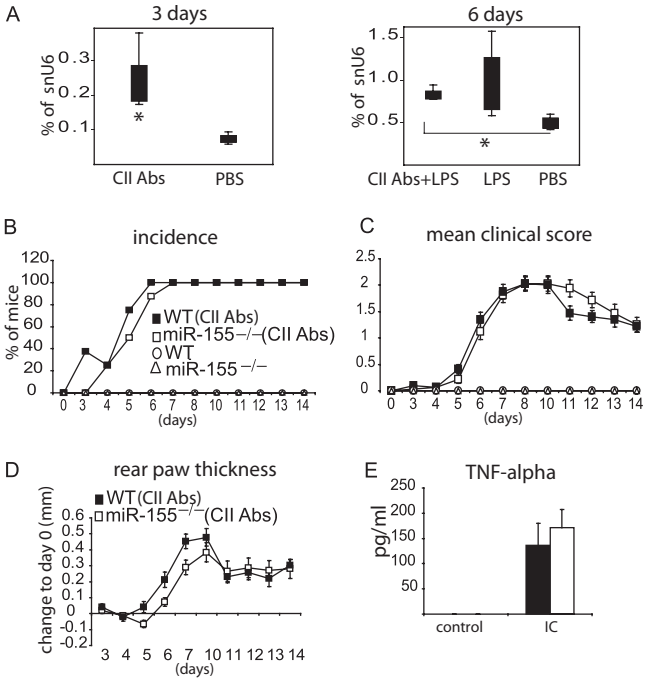

Fig. S5. miR-155 is dispensable for the development of CAIA. On day 0, WT and miR-155$^{-/-}$ mice (n = 8 per group) were injected with the anti-collagen II antibody mixture (8 mg per mouse; i.v.) or PBS. On day 3, 100 μg of *E. coli* LPS was administered i.p. Mice were killed on days 3, 6, and 14. (*A*) miR-155 expression is up-regulated in articular tissue of mice. (*B–D*) The magnitude of CAIA is similar in miR-155$^{-/-}$ mice and in WT animals. Incidence of disease (*B*), mean rear paw clinical score (*C*), and paw size (*D*) were not significantly different. (*E*) miR-155$^{-/-}$ and WT macrophages do not differ in the production of TNF-α after stimulation with immunocomplexes. Macrophages were incubated with PBS (control) or immunocomplexes (IC; 25 μg/mL) for 24 h followed by TNF-α ELISA. Data are means ± SEM or box-and-whisker diagrams. (n = 8). *$P < 0.05$, PBS vs. collagen type 2 antibodies or collagen type 2 antibodies plus LPS. CII Abs, collagen type 2 antibodies.