**Synovial macrophage M1 polarization exacerbates experimental osteoarthritis partially through R-spondin-2**

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**Materials and Methods**

**Supplmentary Figure legends**

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**Human synovium**

Normal human synovium was obtained from victims of road traffic accidents with no history of arthritic diseases (n=7, aged 33.14 ± 4.98 years, four males and three females). Human OA synovium was obtained from patients undergoing total knee replacement surgery (n=9, aged 67.00 ± 3.03 years, one male and eight females). Patients with OA caused by degeneration of articular cartilage were enrolled, and subjects with malignancy, diabetes, or other severe diseases in the previous 5 years were excluded. Human synovium was stained with hematoxylin and eosin (H&E), and by immunofluorescence (IF) and immunohistochemistry (IHC). All human samples were obtained from the Third Affiliated Hospital of Southern Medical University (GuangZhou, China). All patients gave informed consent to use their clinical information for scientific research. The study was approved by the Ethics Committee of the Third Affiliated Hospital of Southern Medical University.

**Transgenic mice and genotyping**

The Lys-MCre mice and Tsc1flox/flox mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA; Jax no.004781 and 005680, respectively). Rheb1flox/flox mice were a generous gift from Professor Bo Xiao, Sichun University. To generate myeloid lineage specific *Tsc1* or *Rheb1* deletion mice, Lys-MCre mice were crossed with Tsc1flox/flox mice and Rheb1flox/flox mice. Mice with myeloid lineage specific deletion of *Tsc1* were termed TSC1KO mice and deletion of *Rheb1* were termed Rheb1KO mice. Littermates carrying Tsc1flox/flox or Rheb1flox/flox without Cre were used as control mice (Control). Routine genotyping of tail DNA was performed according to the Jackson Laboratory’s instructions. All animals were provided with a standard diet and were housed in pathogen-free cages at constant temperature and humidity. The circadian rhythm was maintained at 12 h. All animal experiments were approved by the Southern Medical University Committee Animal Care and Use Committee, and were performed in accordance with the Committee’s guidelines and regulations.

**Experimental OA model**

Eight-week-old C57BL/6 mice were purchased from the Laboratory Animal Centre of Southern Medical University. Eight-week-old male C57BL/6J mice, TSC1KO mice, Rheb1KO mice and control mice were subjected to intra-articular injection of collagenase (CIOA) surgery to induce OA, as previously described.[1](#_ENREF_1)[2](#_ENREF_2) Briefly, 1 U collagenase (C0773, Sigma-Aldrich, St Louis, MO, USA) was injected into the right knee joint twice on alternate days. Eight-week-old male TSC1KO mice and their littermate control mice were also subjected to destabilization of the medial meniscus (DMM) surgery to induce OA. The protocol for DMM surgery has been published by our lab previously.3 4 At postoperative weeks 4 and 8, mice were killed for collection of knee-joint specimens.

**Histological analysis**

Total knee joints were fixed with 4% paraformaldehyde for 24 h, decalcified with 0.5 M EDTA at pH 7.4 for 14 days and subsequently embedded in paraffin. Five-micrometer-thick sections were cut for both hematoxylin and eosin (H&E) and Safranin O/Fast Green (Saf-O) staining. H&E slides were used to evaluate synovial activation by scoring synovial lining cell thickness (0–3)5 6. The medial and lateral compartments of the joint were scored separately, and the sum of the two scores is presented (maximum site score 6). Cartilage degeneration was graded in Safranin-O/Fast Green-stained sections using the Osteoarthritis Research Society International (OARSI)-modified Mankin criteria.[3](#_ENREF_3)Each section was assessed by two blinded, independent graders and the average score was used for statistical analysis.

**Immunohistochemistry and immunofluorescence**

Specimens were prepared as described previously.3 4 After deparaffinization and rehydration, sections were soaked in citrate buffer (10 mM citric acid, pH 6.0) overnight at 60°C to unmask antigen. For immunohistochemical staining, we added 3% hydrogen peroxide for 10 min to inactivate endogenous peroxidase activity. The sections were blocked with 1% goat serum at 37°C for 1 h and stained with primary antibodies overnight at 4°C. For immunohistochemical staining, sections were stained with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and then 3, 3-diaminobenzidine was used to observe the chromogen, with hematoxylin for counterstaining. For immunofluorescence, sections were stained with Alexa 488 or Alexa 594 dye-labeled secondary antibodies (Life Technologies, Carlsbad, CA, USA). Nuclei were labeled with 4, 6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, Waltham, MA, USA) and images were obtained using a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan). Under high magnification imaging, three fields of medial and lateral synovium were selected and the positive staining of synovial intimal lining macrophages was calculated to obtain a mean value. Sections were randomly coded and scored by two blinded observers for three sections per joint.

**Measurement of macrophage polarization and mTORC1 activity in human and mouse synovial tissues**

To visualize macrophage polarization in the joint synovial tissue, immunofluorescence staining for F4/80 (macrophage marker) and iNOS (M1-like macrophage marker) and immunohistochemical staining for CD206 (M2-like macrophage marker) were performed as previously described.7 In brief, for F4/80 and iNOS staining, we incubated slides with the primary antibodies F4/80 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc377009) and iNOS (1:100, Abcam, Cambridge, UK, ab15323) overnight at 4°C, then the slides were washed with PBS and incubated with the following secondary antibodies for 1 hour at room temperature: Alexa Fluor 488 goat anti-mouse IgG2b, Alexa Fluor 488 donkey anti-rabbit IgG (Life Technologies). The slides were then washed with PBS three times, and nuclei were counterstained with DAPI. Images were obtained using a confocal laser scanning microscope (Olympus, Tokyo, Japan). F4/80 and iNOS were labelled in green; nuclei were stained blue. For CD206 staining, slides were incubated with the primary antibodies CD206 (1:100, Abcam, Cambridge, UK, ab64693) overnight at 4°C, then the slides were washed with PBS and incubated with the rabbit anti-mouse HRP secondary antibody (Cell Signaling Technology, Inc., [CST] Danvers, MA, USA; 1:200) for 1 h at room temperature. The sections were then washed in PBS, and visualization was performed with the chromogen diaminobenzidine (Zsbio, Beijing, China) in solution followed by counterstaining with hematoxylin solution. All sections were observed and photographed under an Olympus microscope. In addition, co-localization immunofluorescence staining for pS6 (S235/236) (1:50, CST; #4858) and F4/80 was performed to evaluate the activity of mTORC1 in macrophages. F4/80 was labelled in green and pS6 in red. Under high magnification imaging, three fields of medial and lateral synovium were selected and the positive staining of synovial intimal lining macrophages was calculated to obtain a mean value. Staining was scored in a blinded way by two independent readers. Representative images are shown.

**Cell preparation**

Primary mouse peritoneal macrophages were obtained from the peritoneal exudates of 5- to 8-week-old TSC1KO mice with PBS containing 2% FBS. The purity of macrophages was analyzed by flow cytometry (Beckman Coulter, Brea, CA, USA), using the mouse macrophage marker F4/80. The sorted population was more than 90% pure. Macrophages and their supernatants were collected after stimulation with 10ng/mL LPS (Invivogen, San Diego, CA, USA) for 0, 6, 12, or 24 h.

**rhRspo2 and anti-Rspo2 treatment**

The chondrogenic cell line ATDC5 was cultured as described previously.[3](#_ENREF_3) ATDC5 cells were treated with ITS and conditioned medium (CM) from cultured macrophages from TSC1KO mice or with 10ng/mL rhRspo2 (Sino Biological, Beijing, China, 51078-M05H) or 0.5µg/mL anti-Rspo2 antibody (R&D systems, Minneapolis, MN, USA, MAB3266) or both TSC1KO CM and anti-Rspo2 antibody for 5 or 14 days. For the *in vivo* study, rhRspo2 (1μg/5μL/week) was administered to C57BL/6J mice, while anti-Rspo2 antibody (200ng/5μL/week) was administered to TSC1KO mice with collagenase-induced OA once a week by intra-articular injection for 8 weeks. The control groups were all treated with saline for the same periods.

**Micro-CT analysis**

Micro-computed tomography (micro-CT) of fixed knee joint specimens was performed using a microtomographic imaging system (ZKKS-MCT-Sharp-III scanner, Caskaishen, China). A small field was selected for scanning and corrected for the CT value, with a 70 kV scanning voltage, 30W power, 429 μA current and 5 μm scan thickness. The software 3D-MED 3.0 was used for three-dimensional knee reconstruction and image capture. The region of interest (ROI) was selected from periarticular osteophytes using Mimics 5.0 and marked in red. The ROI size was calculated blinded on all four condyles of the knees (medial and lateral side of the tibia and femur) and the average was used for statistical analysis.

**Western blot analysis**

Cells and cartilage were lysed immediately for 5 min at 95°C in lysis buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 50 mM dithiothreitol, and 0.01% bromophenol blue). Cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Corp., Hercules, CA, USA). Blots were probed with primary antibodies and immunoreactive proteins were revealed using an enhanced chemiluminescence kit (Santa Cruz Biotechnology Inc.).

**Quantitative reverse transcription-polymerase chain reaction**

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). cDNA was reverse transcribed using TaKaRa reverse transcription reagents (TaKaRa Bio Inc., Shiga, Japan) and PCR was performed using Real-Time PCR Mix (TaKaRa) on a light cycler (Roche, Basel, Switzerland) with the following primers: human F4/80 (forward primer 5’-GCA CAT CCA GCC AAA GCA G-3’ and reverse primer 5’-CCA TCT CCC ATC CTC CAC AT-3’ ), human iNOS (forward primer 5’-GTT CTC AAG GCA CAG GTC TC-3’ and reverse primer 5’-GCA GGT CAC TTA TGT CAC TTA TC-3’), human CD206 (forward primer 5’-TGG AGA GGG AAG AGA GTG AAC A-3’ and reverse primer 5’-GCC CAT AAG TGT GCT CTG AA-3’), human GAPDH (forward primer 5’-CTG TTC GAC AGT CAG CCG CAT C-3’ and reverse primer 5’-GCG CCC AAT ACG ACC AAA TCC G-3’), Sox9 (forward primer 5’-TAC CTA CGG CAT CAG CAG CTC-3’ and reverse primer 5’-TTG CCT TCA CGT GGC TTT AAG-3’)，ColII (forward primer 5’-CAC CCT CAA ATC CCT CAA CAA TCA G-3’ and reverse primer 5’-TGT CTT TCG TCT TGC TGG TCC ACC-3’), Acn (forward primer 5’-GAA GGT GAA GGT CGG AGT C-3’ and reverse primer 5’-GAA GAT GGT GAT GGG ATT TC-3’), ColX (forward primer 5’-AAA GCT TAC CCA GCA GTA GG-3’ and reverse primer 5’-ACG TAC TCA GAG GAG TAG AG-3’), Runx2 (forward primer 5’-TCC CCG GGA ACC AAG AAG GCA-3’ and reverse primer 5’-AGG GAG GGC CGT GGG TTC TG-3’). The glyceraldehyde 3-phosphate dehydrogenase gene was used as an endogenous control to normalize for differences in the amount of total RNA.

**ELISA analysis**

We used the Mouse IL-1, IL-6 and TNF- ELISA Kits (Elabscience Biotechnology, Bethesda, MD, USA: #E-EL-M0037c, #E-EL-M0044c and #E-EL-M0049c) to analyze IL-1, IL-6 and TNF- levels in the supernatants of macrophage cultures from TSC1KO and control mice. We used the Mouse Rspo2 ELISA Kit (Cusabio Biotechnology, Wuhan, China, CSB-EL020551MO) to analyze Rspo2 levels in serum and supernatants of macrophage cultures from TSC1KO and control mice. ELISA analysis was performed according to the manufacturers’ instructions.

**Antibodies**

The following antibodies were used: mouse anti-S6 (1:3,000 for WB, Santa Cruz, sc-74459), rabbit anti-MMP13 (1:100 for IHC; Abcam), rabbit anti-Rspo2 (1:100 for IHC; Proteintech, Rosemont, IL, USA, 17781-1-AP), goat anti-Lgr5 (1:100 for IHC; proteintech, 21833-1-AP), mouse anti-α-tubulin (1:3,000 for WB, Rayantibody, RM2007), rabbit anti-β-catenin (1:2000 for WB, 1:200 for IF, CST, #8480).

**Statistical analysis**

All experiments were performed in duplicate or triplicate and observed by independent observers. Differences between two groups were analyzed using Student’s *t*-test while those among three groups were analyzed by one-way analysis of variance (ANOVA) and Tukey’s multiple comparison test. All statistical analyses were performed with Graph Pad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). The results are presented as the mean ± standard error (SEM), and *p*<0.05 was considered statistically significant.

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**Supplmentary Figure legends**

**Supplementary Figure 1. Macrophage polarization in synovial tissue from OA patients and OA mice.** (A) Quantitative PCR analysis of F4/80, iNOS and CD206 mRNA in normal (*n*=7) and OA (*n*=9) human synovial tissues. Student’s *t*-test. NS: not significant. \*\*P<0.01. (B) Representative images of knee joint and ACL of CIOA mice following H&E staining. Boxed area is enlarged in Figure 1D. Data are shown as mean±SEM. F, Femur; T, Tibia; ACL, Anterior cruciate ligament. Black solid arrows signify the injury of ACL. Scale bar, 500 μm or 50 μm.

**Supplementary Figure 2. Macrophage polarization and mTORC1 activity in synovial tissue from young and aged mice.** (A) Representative images of H&E staining in synovium from mice collected at 3 and 24 months of age. Scale bars, 50 μm, 500 μm. (B) Quantification of synovitis score and hyaline cartilage/calcified cartilage (HC/CC) in synovium from mice collected at 3 and 24 months of age. *n*=8. Student’s *t*-test. \*\*P<0.01. (C) Representative images of co-immunostaining of F4/80 and pS6 (C-top), immunofluorescence of iNOS (C-middle), immunohistochemistry of CD206 (C-bottom) in synovium from mice collected at 3 and 24 months of age. Scale bar, 50 μm. (D) Quantification of pS6-, iNOS- and CD206-positive macrophages as a proportion of total macrophages in synovium from mice collected at 3 and 24 months of age. *n*=8. Student’s *t*-test. \*\*P<0.01. Boxed area is enlarged in the bottom right corner. pS6, red; F4/80, green; iNOS, green; DNA, blue. Data are shown as mean ± SEM.

**Supplementary Figure 3. Generation and phenotypes of TSC1KO.** (A) Genotyping the offspring after mating transgenic Cre and loxp mice. Original image of agarose gel electrophoresis. Lyz(-)-Tsc1flox/flox: control mice, Lyz(+)-Tsc1flox/flox: knockout mice. (B) General phenotypes of control and TSC1KO mice at 8 weeks after birth. (C) Graph of control and TSC1KO mouse body weights from postnatal week 1 to week 8. *n*=8. (D) Graph of control and TSC1KO mouse body length at 8 weeks of age. *n*=8. Student’s *t*-test. NS, not significant. (E) Images (left) and quantification (right) of the limbs of control and TSC1KO mice at 8 weeks of age. *n*=8. Student’s *t*-test. NS, not significant. (F) Representative images of H&E-stained articular cartilage and growth plates in tibias from 8-week-old control and TSC1KO mice. Scale bars, 50 μm. (G) Quantification of HC/CC and the lengths of proliferative zone (PZ) and hypertrophic zone (HZ) in the growth plates of tibias from 8-week-old control and TSC1KO mice. *n*=8. Student’s *t*-test. NS, not significant. (H) Representative images of joint ACL from TSC1KO and control mice at 4 weeks and 8 weeks after CIOA surgery. Scale bars, 200 μm. (I) Representative images of H&E-stained synovium from TSC1KO and control mice at 4 weeks and 8 weeks after CIOA surgery. Scale bars, 50 μm. (J) Quantification of synovitis score in synovium from TSC1KO and control mice at 4 weeks (*n*=10) and 8 weeks (*n*=9) after CIOA surgery. Student’s *t*-test. \*P<0.05. Data are shown as means ± SEM.

**Supplementary Figure 4. Synovial macrophage M1 polarization promotes DMM-OA development in mice.** Representative images of Safranin O/fast green staining of joints from TSC1KO and control mice at 4 and 8 weeks post DMM surgery, and quantitative analysis of OARSI scale. *n*=9. Student’s *t*-test. \*P<0.05. Data are shown as mean ± SEM.

**Supplementary Figure 5. Generation and phenotypes of Rheb1KO mice.** (A) Genotyping the offspring after mating transgenic Cre and loxp mice. Original image of agarose gel electrophoresis. Lyz(-)-Rheb1flox/flox: control mice, Lyz(+)-Rheb1flox/flox: knockout mice. (B) General phenotypes of control and Rheb1KO mice at 8 weeks after birth. (C) Graph of control and Rheb1KO mouse body weights from postnatal week 1 to week 8. *n*=8. (D) Graph of control and Rheb1KO mouse body length at 8 weeks of age. *n*=8. Student’s *t*-test. NS, not significant. (E) Images (left) and quantification (right) of the limbs of control and Rheb1KO mice at 8 weeks of age. *n*=8. Student’s *t*-test. NS, not significant. (F) Representative images of H&E-stained articular cartilage and growth plates in tibias from 8-week-old control and Rheb1KO mice. Scale bars, 50 μm. (G) Quantification of HC/CC and the lengths of the proliferative zone (PZ) and hypertrophic zone (HZ) in the growth plates of tibias from 8-week-old control and Rheb1KO mice. *n*=8. Student’s *t*-test. NS, not significant. (H) Representative images of joint ACL from Rheb1KO and control mice at 4 weeks and 8 weeks after CIOA surgery. Scale bars, 200 μm. (I) Representative images of H&E-stained synovium from Rheb1KO and control mice at 4 weeks and 8 weeks after CIOA surgery. Scale bars, 50 μm. (J) Quantification of synovitis scores in synovium from Rheb1KO and control mice at 4 weeks (*n*=11) and 8 weeks (*n*=8) after CIOA surgery. Student’s *t*-test. \*P<0.05. Data are shown as means ± SEM.

**Supplementary Figure 6. M1 polarized macrophages produce inflammatory cytokines and Rspo2.** The primary macrophages from TSC1KO mice were stimulated with 10ng/mL LPS for 0, 6, 12, 24 h. (A) Quantitative PCR analysis of IL-1, IL-6 and TNF-α mRNA in primary macrophages from TSC1KO mice. One-way ANOVA and Tukey’s multiple comparison test. \*\*P<0.01. (B) IL-1, IL-6 and TNF-α concentrations assessed by ELISA in supernatant of cultured macrophages from TSC1KO mice. One-way ANOVA and Tukey’s multiple comparison test. \*\*P<0.01. (C) Quantitative PCR analysis of Rspo2 mRNA in primary macrophages from TSC1KO mice. One-way ANOVA and Tukey’s multiple comparison test. \*\*P<0.01. (D) Rspo2 concentrations assessed by ELISA in supernatant of macrophage culture from TSC1KO mice; *n*=8 per group. One-way ANOVA and Tukey’s multiple comparison test. \*\*P<0.01. Data are shown as mean±SEM.

**Supplementary Figure 7. M1 polarized macrophages exacerbate experimental OA partially through Rspo2.** (A and B) Quantitative PCR analysis of Sox9, Col2a1, Acan, Col10a1, Runx2 and toluidine blue staining in ATDC5 cells treated with rhRspo2 or TSC1KO CM or both TSC1KO CM and anti-Rspo2 antibody. *n*=6 per group. One-way ANOVA and Tukey’s multiple comparison test. \*P<0.05. \*\*P<0.01. (C) Western blot of β-catenin in ATDC5 cells treated with rhRspo2 or TSC1KO CM or both TSC1KO CM and anti-Rspo2 antibody.