



Title	Role of Chondrocytes in the Development of Rheumatoid Arthritis Via Transmembrane Protein 147-Mediated NF-kappa B Activation
Author(s)	Ota, Mitsutoshi; Tanaka, Yuki; Nakagawa, Ikuma; Jiang, Jing-Jing; Arima, Yasunobu; Kamimura, Daisuke; Onodera, Tomohiro; Iwasaki, Norimasa; Murakami, Masaaki
Citation	Arthritis & rheumatology, 72(6), 931-942 https://doi.org/10.1002/art.41182
Issue Date	2020-06
Doc URL	http://hdl.handle.net/2115/81699
Rights	This is the peer reviewed version of the following article: Ota, M., Tanaka, Y., Nakagawa, I., Jiang, J. J., Arima, Y., Kamimura, D., Onodera, T., Iwasaki, N. and Murakami, M. (2020), Role of Chondrocytes in the Development of Rheumatoid Arthritis Via Transmembrane Protein 147-Mediated NF κ B Activation. <i>Arthritis Rheumatol</i> , 72: 931-942, which has been published in final form at https://doi.org/10.1002/art.41182 . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.
Type	article (author version)
Additional Information	There are other files related to this item in HUSCAP. Check the above URL.
File Information	Arthritis Rheumatol 72 931.pdf



[Instructions for use](#)

1 **Chondrocytes play a role in the development of rheumatoid arthritis via TMEM147-mediated**
2 **NF-κB activation**

3
4 Mitsutoshi Ota^{1, 2}, Yuki Tanaka¹, Ikuma Nakagawa¹, Jing-Jing Jiang¹, Yasunobu Arima¹, Daisuke
5 Kamimura¹, Tomohiro Onodera², Norimasa Iwasaki², Masaaki Murakami¹.

6
7 ¹ Division of Molecular Psychoimmunology, Institute for Genetic Medicine, Graduate School of
8 Medicine, Hokkaido University, Sapporo, Japan

9 ² Department of Orthopaedic Surgery, Hokkaido University Graduate School of Medicine, Sapporo,
10 Japan

11 Address corresponded to:

12 Masaaki Murakami
13 N15, W7, Kita-Ku, Sapporo, Hokkaido 060-0815, Japan
14 Phone : +81-11-706-5120
15 Fax : +81-11-706-7542
16 e-mail : murakami@igm.hokudai.ac.jp
17
18

19 **ABSTRACT**

20 **Objective** We previously reported that the co-activation of NF- κ B and STAT3 in non-immune cells
21 including synovial fibroblasts enhances the expression of NF- κ B target genes, playing a role in
22 chronic inflammation including rheumatoid arthritis (RA). Here we examined the role of NF- κ B
23 activation in chondrocytes to understand the pathogenesis of RA. Furthermore, we investigated
24 TMEM147 as a representative NF- κ B activator in chondrocytes.

25
26 **Methods** Clinical samples from RA patients were analyzed by immunohistochemistry. Samples from
27 polydactyly patients served as a control. The functional contribution of chondrocytes and TMEM147
28 to arthritis was examined in several mouse RA models. In vitro experiments including qPCR, RNA
29 interference, immunoprecipitation and confocal microscopy were performed to investigate the
30 mechanism of action of TMEM147 in chondrocytes.

31
32 **Results** Samples from RA patients and from the RA models showed co-activation of NF- κ B and
33 STAT3 in chondrocytes. This co-activation induced a synergistic expression of NF- κ B targets in vitro.
34 Chondrocyte-specific deletion of STAT3 significantly suppressed the development of
35 cytokine-induced arthritis. TMEM147 was highly expressed in chondrocytes from RA patients and
36 the RA models. Gene silencing of TMEM147 or anti-TMEM147 antibody treatment inhibited the
37 cytokine-mediated activation of NF- κ B in vitro and suppressed the cytokine-induced arthritis in vivo.
38 Mechanistically, TMEM147 molecules acted as a scaffold protein for a NF- κ B complex that included
39 breakpoint cluster region and casein kinase 2 to enhance NF- κ B activity.

40
41 **Conclusion** These results suggest that chondrocytes play a role in the development of RA via
42 TMEM147-mediated NF- κ B activation and provide a novel therapeutic strategy for RA.

43
44 **KEYWORDS**

45 Rheumatoid arthritis, Chondrocytes, Inflammation, Cytokines, NF- κ B, TMEM147

47 INTRODUCTION

48 Cartilage is an essential component of the synovial joints and is composed of chondrocytes. Cartilage
49 shows extremely high durability in healthy conditions, but also has poor natural healing ability.
50 Therefore, once it is damaged, it gradually deteriorates to ultimately dysfunction¹. Rheumatoid
51 arthritis (RA) is a chronic inflammatory disease that causes joint destruction². In RA, cartilage is
52 considered a target tissue for synovial inflammation³. Indeed, during RA development, cytokines
53 expressed from immune cells and fibroblast-like synoviocytes damage the cartilage tissue by
54 promoting the production of matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS
55 families) from the chondrocytes^{4 5}. Chondrocytes secrete MMPs and ADAMTS families after
56 stimulation with CXCL6 and FGF-2, leading to their dysfunction {Kawaguchi, 2008 #38;Pap, 2015
57 #9;Sanchez, 2017 #10}. On the other hand, it is reported that heterozygous knockout of NF-κB p65
58 or ADAMTS5 deficiency in chondrocytes retards the development of an osteoarthritis model via
59 catabolic inhibition or of an antigen-induced arthritis model, respectively {Kobayashi, 2016
60 #47;Stanton, 2005 #48}. At the same time, the homozygous knockout of NF-κB p65 in chondrocytes
61 enhances the development of osteoarthritis via chondrocyte apoptosis⁶. As a result, the role of the
62 NF-κB pathway in chondrocytes remains controversial during RA development.

63 Although inflammation is an important biological defense, chronic inflammation is known to cause
64 metabolic diseases, neurodegenerative diseases and autoimmune diseases including RA⁶⁻⁸. As a key
65 molecular mechanism in chronic inflammation, we previously reported the inflammation amplifier,
66 which was originally termed the IL-6 amplifier^{9 10}. The inflammation amplifier is a hyper NF-κB
67 activation machinery in non-immune cells including synovial fibroblasts induced by the
68 simultaneous activation of NF-κB and STAT3. It induces a massive and sustained production of
69 NF-κB target genes, including IL-6, chemokines, and growth factors, which is critical for the
70 development of various disease models including RA^{9 10}. Furthermore, we have shown that the
71 co-activation of NF-κB and STAT3, which is evidence of activation of the inflammation amplifier, is
72 observed in clinical specimens from patients with inflammatory diseases^{11 12}. Additionally, the
73 expression of target molecules of the inflammation amplifier is higher in the serum of patients with
74 RA or multiple sclerosis {Murakami, 2013 #6;Harada, 2015 #3}. These findings suggest a new
75 therapeutic strategy based on the amplifier. Accordingly, we conducted a shRNA-based genome wide
76 screening, in which we identified more than 1,000 genes involved in activation of the inflammation
77 amplifier⁸. In particular, we reported that BCR, which is known to form the oncogenic fusion protein
78 BCR-Abl¹³, interacts with the α subunit of casein kinase II (CK2α), leading to the phosphorylations
79 of BCR at tyrosine 177 and NF-κB p65 at serine 529 to transcriptionally activate NF-κB p65 in the
80 nucleus following TNF-α stimulation. Thus, the formation of the BCR-CK2α-NF-κB p65 complex in
81 the cytoplasmic region plays a role in nuclear function of NF-κB¹⁴.

82 In the present study, we focused on transmembrane protein 147 (TMEM147) as a positive regulator
83 of NF-κB, because of its high expression in chondrocytes in the inflamed area. TMEM147 is a
84 seven-transmembrane protein whose functions are not well known¹⁵. We here demonstrated the
85 importance of chondrocytes in amplifying inflammatory responses during RA development based on
86 the following six observations: (i) chondrocytes and a chondroprogenitor cell line, ATDC5,
87 synergistically expressed NF-κB targets after stimulation with TNF-α or IL-17 plus IL-6, (ii)
88 chondrocytes in RA patients and in mouse models of RA showed the simultaneous activation of
89 NF-κB and STAT3, (iii) chondrocytes deficient of STAT3 suppressed the development of the RA
90 model, (iv) the deficiency or blockade of TMEM147 molecules suppressed the expression of NF-κB
91 targets in vitro and in vivo, (v) TMEM147 acted as a scaffold protein for the BCR/CK2α-mediated
92 NF-κB activation pathway, and (vi) anti-TMEM147 antibody injections in the joints suppressed the
93 development of the RA model. These findings reveal a novel role for chondrocytes in RA

96 development via TMEM147-dependent NF-κB activation.

97

98

MATERIALS AND METHODS

99

Mouse Strains

100 C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). The F759 (gp130^{F759/F759})
101 knock-in mouse line was previously established¹⁶. Col2a1-Cre mice (B6; SJL-Tg [Col2a1-Cre]
102 1Bhr/J) were obtained from the Jackson Laboratory (Bar Harbor, ME) and crossed with
103 STAT3flox/flox mice that were provided by S. Akira (Osaka University, Japan)¹⁷. All animal
104 experiments performed in this study were approved by Hokkaido University, Institute for Genetic
105 Medicine, Japan, and all mice were housed and maintained under specific pathogen-free conditions
106 according to the institute's animal care guidelines. Animal experiments were performed following
107 the guidelines of the Institutional Animal Care and Use Committees of Hokkaido University. The
108 protocols for animal experiments were approved by the Institutional Animal Care and Use
109 Committees of Hokkaido University.

110

111

Spontaneous RA models

112 F759 mice^{16 18} (12-months old) were euthanized, and their entire ankle joints were dissected for
113 examinations.

114

115

Cytokine-induced arthritis models

116 Human IL-6 (100 ng, Toray Industries, Tokyo, Japan), mouse IL-17A (100 ng, R&D Systems, Tokyo,
117 Japan) or saline was injected into the ankle joints of F759 mice, as described previously¹⁹. In some
118 experiments, lentivirus particles carrying short hairpin RNA (shRNA) specific for TMEM147
119 (Sigma-Aldrich, Tokyo, Japan) or a scrambled sequence (Sigma-Aldrich) were injected into the
120 ankle joints of the mice^{8 14 19-23}. Anti-TMEM147 antibodies (Sigma-Aldrich) were injected into the
121 ankle joints of mice at a dose of 2 µg/day. The same dosage of rabbit IgG (Santa Cruz Biotechnology,
122 Tokyo, Japan) served as a control. Clinical signs of arthritis were evaluated, as described previously
123¹⁹. In brief, the severity of the arthritis was determined based on restricted mobility of the ankle joints
124 with a scale of 0–3, where 0 indicates no change; 1, mild change; 2, medium change; and 3, severe
125 change. Averages for a single point in one leg ankle joint from each mouse were used. The mice were
126 euthanized, and their entire ankle joints were dissected for the examinations.

127

128

Spontaneous OA models

129 C57BL/6 mice²⁴ (24 months old) were euthanized, and their entire ankle joints were dissected for
130 the examinations.

131

132

Collagen-induced arthritis models

133 C57BL/6 mice (8 months old) were immunized with CFA plus type I collagen peptide (sequences?),
134 and their entire ankle joints were dissected for the examinations.

135

136

Clinical specimens

137 RA patients (n = 6) and OA patients (n = 5) undergoing total knee arthroplasty (TKA) surgery, and
138 polydactyly patients (control; n = 5) undergoing reconstruction surgery at Hokkaido University
139 Hospital, Sapporo, Japan, were recruited to the study after written informed consent (Supplementary
140 Table 1). Every patient with RA fulfilled the American College of Rheumatology criteria for the
141 classification of RA {Aletaha, 2010 #42}, and every patient with OA was classified with
142 Kellgren-Lawrence grade 3 or 4 {Kellgren, 1957 #46}. Sample collection, processing, storage and
143 subsequent experimental procedures were carried out in compliance with Human Tissue Authority
144 guidelines under the Human Tissue Act (2004). The study protocol was approved by the Human
145 Ethics Committee of Hokkaido University Hospital and was conducted in accordance with the
146 Declaration of Helsinki Principles. All patient identifiers were removed from the data.

147

148 **Cells and stimulation conditions**

149 A chondroprogenitor cell line, ATDC5, was obtained from Riken Cell Bank (Ibaraki, Japan). Primary
150 murine chondrocytes were prepared from newborn mice, as described previously²⁵. Primary human
151 chondrocytes were sourced from non-pathologic articular cartilage (Cell Applications Inc., San
152 Diego, CA, USA). Cells were plated in 96-well flat-bottom plates or 100 mm dishes and stimulated
153 with human IL-6 plus soluble human IL-6R α (100 ng/mL each; Toray Industries) and/or mouse or
154 human IL-17A (50 ng/mL; R&D Systems) as well as mouse or human TNF- α (50 ng/ml; PeproTech,
155 Tokyo, Japan) after 2 hr serum starvation. Anti-TMEM147 antibody (20 ng/mL; Sigma-Aldrich) was
156 added to some experiments. Mouse cells are responsive to human IL-6 which allowed us to measure
157 mouse IL-6 in the culture supernatant after stimulation with human IL-6 and mouse IL-17A using
158 mouse-specific IL-6 ELISA (eBioscience and BD Biosciences, Tokyo, Japan).

159

160 **TMEM147 shRNA knockdown cells**

161 ATDC5 cells were cultured on day 0 in a 96-well flat-bottom plate (1 x 10³ cells/well) in 100 μ L of
162 DMEM/F-12 (1:1) with 2 mM L-Glutamine containing 5% FBS. The medium was replaced on day 1
163 with DMEM/F-12 (1:1) containing lentivirus carrying 1 μ L of candidate-specific shRNA
164 (TMEM147 shRNA, TRCN0000174842; non-target shRNA, SHC002; Sigma-Aldrich), 5% FBS and
165 8 μ L/mL Polybrene. 200 μ L of DMEM/F-12 (1:1) containing 5% FBS and 5 μ g/mL puromycin was
166 added to each well on day 2. Cells that survived drug selection with puromycin were designated as
167 TMEM147 knockdown cells and the knockdown efficiency was verified by qPCR and Western
168 blotting.

169

170 **Human small interfering RNAs**

171 Primary human chondrocytes were cultured on day 0 in a 96-well flat-bottom plate (7 x 10³
172 cells/well) in 100 μ l of Human Chondrocyte Media (Cell Applications Inc.). A mixed solution of
173 Opti-MEM (18 μ L/well, Thermo Fisher Scientific, Kanagawa, Japan), 5 μ M small interfering RNAs
174 (siRNAs) and Lipofectamine RNAiMAX (0.28 μ L/well, Thermo Fisher Scientific) was added into
175 the culture medium on day 1. The medium was replaced on day 2 with Human Chondrocyte Media
176 (Cell Applications Inc.). The knockdown efficiency was verified by qPCR, and the sequences for the
177 sense oligonucleotides of the most effective knockdown constructs were as follows: human
178 siTMEM147 (s20403; Thermo Fisher Scientific) and human non-target (SIC-001; Sigma-Aldrich).

179

180 **Plasmids**

181 The Flag-tagged murine TMEM147 expression vector was constructed as follows.
182 TMEM147-encoding cDNA was synthesized from the total cellular RNA of ATDC5 cells using KOD
183 FX (TOYOBO, Osaka, Japan) for RT-PCR with a primer pair. The cDNA was electrophoresed on
184 2.5% agarose gel and extracted. The cDNA and pCMV-Tag2B were treated with restriction enzymes
185 (BamH I, Sal I, High buffer, TaKaRa) at 37 °C for 2.5 hr and ligated with ligation buffer (Ligation
186 high Ver 2, TOYOBO) at 16 °C for 2 hr. The prepared vector was transfected into competent cells
187 (ECOS DH5 α , NIPPON GENE) and cultured in 100 mm LB Agar plate containing kanamycin (20
188 μ g/mL) overnight at 37 °C. The colonies generated by drug selection with kanamycin were picked up
189 and cultured in 100 mL LB medium containing kanamycin (20 μ g/mL) overnight at 37 °C in an
190 incubator shaker (EYLA, Tokyo, Japan). The GenElute HP Plasmid Midiprep Kit (Sigma-Aldrich)
191 was used to extract plasmid DNA from the culture medium. The presence of the intended fragment
192 without any unexpected mutations was confirmed by DNA sequencing with a BigDye Terminator
193 v3.1 Kit (Applied Biosystems, Tokyo, Japan). All primers are listed in Supplementary Table 2.

194

195 **Antibodies**

The following antibodies (Abs) were used for western blotting, immunoprecipitation, qPCR, ELISA, confocal microscopy, immunohistochemistry, flow cytometry, or joint injection: anti-TMEM147 (sc-138814, Santa Cruz Biotechnology), anti-TMEM147 (generated by immunizing rabbit with peptide containing amino acids 121-136 VGARGIEFDWKYIQMSC, Sigma-Aldrich), anti-NF- κ B p65 (sc-372, Santa Cruz Biotechnology), anti-phospho-p65 S536 (#3033, Cell Signaling Technology, Tokyo, Japan), anti-phospho NF- κ B p65 S529 (ab47395, Abcam, Tokyo, Japan), anti-phospho NF- κ B p65 S276 (SAB4504488, Sigma-Aldrich), anti-phospho-stat3 (#9145, Cell Signaling Technology), anti-I κ B α (#4814, Cell Signaling Technology), anti-phospho-I κ B α (#9246, Cell Signaling Technology), anti-BCR (#3902, Cell Signaling Technology), anti-phospho-BCR Y177 (#3901, Cell Signaling Technology), anti-CK2 α (#2656, Cell Signaling Technology), anti-phospho-CK2 α (SAB4300628, Sigma-Aldrich), anti- α -tubulin (T5168, Sigma-Aldrich), anti-FLAG M2 (F1804, Sigma-Aldrich), rabbit IgG (#3900, Cell Signaling Technology), anti-rabbit IgG (sc-2004, Santa Cruz Biotechnology), anti-mouse IgG (sc-2314, Santa Cruz Biotechnology), anti-goat IgG (sc-3851, Santa Cruz Biotechnology), Alexa Fluor 488 goat anti-rabbit IgG (H+L) (A11055, Life technologies, Tokyo, Japan) and Hoechst 33342 trihydrochloride trihydrate (H3570, Life technologies).

Quantitative real-time PCR (qPCR)
Total RNA was isolated from the cells using the SuperPrep Cell Lysis Kit for qPCR (Toyobo, Osaka, Japan) and was isolated from the tissue using ISOSPIN Cell & Tissue RNA (NIPPON GENE, Toyama, Japan). cDNAs were made using M-MLV Reverse Transcriptase (Promega, Tokyo, Japan). mRNA levels were quantified by qPCR using ABI Prism 7300 fast real-time PCR system (Applied Biosystems, Tokyo, Japan) and SYBR Green FAST qPCR master mix (Kapa Biosystems, Woburn, MA). The conditions for real-time PCRs were 40 cycles at 94 °C for 15 sec followed by 40 cycles at 60 °C for 60 sec. The relative mRNA expression levels were normalized to the levels of HPRT or GAPDH mRNA expression. All primers are listed in Supplementary Table 2.

Enzyme-linked immunosorbent assay (ELISA) and MTT assay
Cells were plated in 96-well flat-bottom plates and cultured overnight on day 0. On day 1, cell stimulation was conducted for 24 hr under the conditions described above. The culture supernatant was collected and mouse IL-6 concentrations were determined using ELISA kits specific for mouse IL-6 (BD Biosciences). 100 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-containing DMEM (500 μ g/mL) was added to the cells after ELISA, and the color reaction was carried out by culturing at 37 °C for 2 hr. After removal of the culture supernatant, the produced formazan was dissolved in 100 μ L DMSO. The absorbance was measured with a Model 680 Microplate Reader (BIO RAD, Tokyo, Japan).

Western blotting
Mock (non-target control), TMEM147 knockdown cells and cells which were forced to express TMEM147 were lysed with HBST (0.5) lysis buffer (0.5% TritonX-100, 150 mM NaCl, 10 mM HEPES [pH7.4]) containing 1/100 volume of protease inhibitor mixture, phosphatase inhibitor mixture 2 and phosphatase inhibitor mixture 3 (Sigma-Aldrich). The protein lysates were heated only to 60 °C to avoid the aggregation of TMEM147. 20 μ g of total protein was run on 5-20% SDS-PAGE (Wako, Tokyo, Japan). After transfer to a polyvinylidene fluoride membrane (Pall Corporation, Port Washington, NY), immunoblotting was performed according to the manufacturer's protocol.

Immunoprecipitation
The protein lysates, which were adjusted to 1 mg/mL for immunoprecipitation, were assessed

245 pre-clear with 30 µL of protein G Sepharose to remove non-specific binding proteins (17-0618-02,
246 GE Healthcare, Tokyo, Japan) and incubated for 1 hr at 4 °C with gentle agitation. The supernatant
247 was mixed with 30 µL of anti-Flag beads (A2220, Sigma-Aldrich, Tokyo, Japan), followed by
248 rotation for 2 hr at 4 °C. For the detection of p65 S529 phosphorylation, the supernatant was mixed
249 with 2 µg of anti-p65 Ab, which was immobilized on protein G Sepharose, followed by rotation for 2
250 hr at 4°C. The immunoprecipitates were eluted with 3X Flag peptide (F4799, Sigma-Aldrich) or 2X
251 SDS-PAGE loading buffer, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride
252 membrane followed by Western blotting with the antibodies, as previously described (references).
253

254 **Luciferase Assay**

255 pGL4.32[luc2P/NF-κB-RE/Hygro] or IL-6-RE cloned into pGL4.20[luc2P/Puro] vector and pRL-TK
256 vector (Promega, Tokyo, Japan), and pCMV-Tag2B vector containing cDNA for TMEM147 were
257 transiently co-transfected into HEK293T cells by using polyethylenimine (PEI). Cells cultured in
258 96-well flat-bottom plates were harvested after 24 hr transfection and stimulated with TNF-α (50
259 ng/mL; PeproTech, Tokyo, Japan) for 6 hr. Luciferase activities of total cell lysates were measured
260 using a Dual-Luciferase reporter assay system (Promega, Tokyo, Japan) and Glomax-Multi
261 Detection System (Promega, Tokyo, Japan).

262 **Confocal laser scanning microscopy**

263 Mock (non-target control) and TMEM147 knockdown cells were stimulated with TNF-α for 0, 15,
264 and 30 min on µ-Slides (Ibidi, Martinsried, Germany). The stimulated cells were fixed and
265 permeabilized with a Cytofix/Cytoperm Kit (BD Biosciences), and incubated with rabbit anti-p65 (1
266 µg/mL) for 1 hr at 4 °C. After washing, the cells were incubated with anti-rabbit Alexa Fluor
267 488-conjugated secondary Ab (10 µg/mL) and Hoechst 33342 nuclear stain (1 µg/mL) for 1 hr at
268 4 °C. Cells were then observed by confocal microscopy (LSM5 Pascal system, Zeiss, Tokyo, Japan).
269

270 **Cell surface flow cytometry analysis**

271 The cell surface expression of TMEM147 on ATDC5 cells was detected using generated
272 anti-TMEM147 Ab (1 µg/mL). The secondary detection antibody used was anti-rabbit Alexa Fluor
273 488-conjugated Ab (10 µg/mL). Control IgG was used at the same concentrations. Non-specific
274 binding was blocked with the Fc block (10-20 µg/mL; BD Biosciences) before antibody staining. For
275 all flow cytometry experiments, cells were stained for 30 min at 4 °C in the dark and washed three
276 times with flow cytometry buffer (PBS + 1% FBS). Samples were analyzed using CyAn™ ADP
277 (BECKMAN COULTER, Tokyo, Japan). Data were analyzed with FlowJo software (BD
278 Biosciences).
279

280 **Statistical analysis**

281 Student's t tests (two-tailed) were used for the statistical analyses of differences between two groups.
282 One-way ANOVA with Dunnett's post hoc analysis was used for multiple comparisons. Wilcoxon
283 rank-sum test was used for the statistical analyses of clinical scores of the arthritis model. *P* values
284 less than 0.05 were considered significant (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001).
285

287

RESULTS

288

Enhanced NF-κB activation in chondrocytes plays an important role in the pathogenesis of RA mouse models

289

We first investigated whether the inflammation amplifier is activated in the chondrocytes of synovial joints. Activation was monitored by the enhanced expression of NF-κB targets such as IL-6^{8 9 11 12 14 19-23 27 28}. Murine primary chondrocytes were prepared²⁵ and stimulated with IL-6, IL-17A and/or TNF-α. IL-6 mRNA and protein levels were significantly increased in chondrocytes (Fig. 1A and 1B). The expression of MMP13 and ADAMTS5 also increased after cytokine stimulation in chondrocytes (Fig. S1). The mouse chondroprogenitor cell line ATDC5 also showed the synergistic production of IL-6 after cytokine treatment (Fig. S2).

290

We then investigated whether the activation of NF-κB and STAT3 can be observed in inflamed joints. We first employed F759 knock-in mice (12-months old) as a RA model, because they spontaneously develop RA-like arthritis with age due to exaggerated IL-6 signaling caused by the lack of a SOCS3-binding site on the IL-6 receptor gp130^{16 18}. In older F759 mice, cartilage tissue denaturation was observed with Safranin-O staining, and the activation of NF-κB and STAT3 was confirmed in the chondrocytes (Fig. 1C-1E). Consistent with this observation, bone destruction was clearly observed by using computed tomography (CT) analysis (Fig. S3A and S3H), and the knee joints were similarly affected (Fig. S4A-S4C). F759 mice with chondrocyte-specific deletion of STAT3 were generated using Col2a1 promoter-driven cre mice, which prevents the co-activation of NF-κB and STAT3 in chondrocytes. Because RA-like arthritis in F759 mice is dependent on the age-dependent increase of IL-6 and IL-17 from Th17 cells²⁷, direct articular injections of IL-6 and IL-17 accelerated the arthritis induction to within two weeks^{8 9 14 19-23}. This cytokine-induced arthritis was significantly suppressed in Col2a1-STAT3^{f/f} F759 mice (Fig. 1F). Synovial hyperplasia, cartilage degeneration, and the activation of NF-κB and STAT3 were suppressed in Col2a1-STAT3^{f/f} F759 mice according to histological analysis (Fig. S5A-S5C). In the cytokine-induced arthritis model, bone erosion was not detected in 8-week-old mice, most likely because of their young age (Fig. S3). Soft tissue swelling was suppressed, and bone destruction was reduced in 12-month-old Col2a1-STAT3^{f/f} F759 mice (Fig. S3D, S3E, S3H and S3I). Thus, enhanced NF-κB activation in chondrocytes has a crucial pathogenic role in the development of arthritis in RA models.

316

Enhanced activation of NF-κB in chondrocytes was observed in RA patients

317

We next investigated whether activation of the inflammation amplifier also occurs in RA patients. The expression of IL-6 mRNA was significantly increased in human primary chondrocytes stimulated with IL-6 plus IL-17A or TNF-α (Fig. 2A). The joint cartilage of RA patients was remarkably denatured, as judged by Safranin-O staining, and the activation of NF-κB and STAT3 was significantly enhanced in the chondrocytes (Fig. 2B and 2C).

323

TMEM147 is critical for NF-κB activation in chondrocytes

324

TMEM147, a candidate gene marker of inflammation amplifier activation⁸, was clearly increased in chondrocytes in the ankle joints of F759 mice as well as RA patients (Fig. 3A and 3B). Similar results were observed in the knee joints of a mouse RA model and Col2a1-STAT3^{f/f} F759 mice (Fig. S4D and S5D). ATDC5 cells with TMEM147 knockdown showed less IL-6 expression after IL-6 plus IL-17 stimulation (Fig. 3C-3F), while the forced expression of TMEM147 increased the reporter activity of NF-κB and IL-6 promoters (Fig. 3G and 3H) and enhanced IL-6 expression (Fig. S6). TMEM147 knockdown suppressed the expression other NF-κB target genes including CCL20 and CXCL1, but not a STAT3 target, STAT3 itself²⁹ (Fig. 3I), suggesting that TMEM147 controls the NF-κB pathway. Importantly, TMEM147 played a role in NF-κB activation in human primary chondrocytes (Fig. 3J-3L). Moreover, the clinical scores of the cytokine-induced arthritis was significantly suppressed by TMEM147 knockdown in the ankle joints (Fig. 3M). Synovial

336 hyperplasia and cartilage degeneration were suppressed in TMEM147 knockdown mice (Fig. S7).
337 Soft tissue swelling was suppressed in TMEM147 knockdown mice, and bone destruction was not
338 observed (Fig. S3F, S3G). These results suggested that TMEM147 is a positive regulator of NF- κ B
339 activation in chondrocytes in vitro and in vivo.
340

341 **TMEM147 acts as a scaffold protein for the BCR-CK2 α complex**

342 We next investigated which step(s) of the NF- κ B signaling pathway is positively regulated by
343 TMEM147 molecules. The phosphorylation of NF- κ B p65 at S536 as well as the phosphorylation
344 and degradation of I κ B α were not affected in TMEM147 knockdown cells after TNF- α stimulation
345 (Fig. 4A). Consistently, there was no significant change in the nuclear translocation of NF- κ B p65
346 between mock and TMEM147 knockdown cells (Fig. 4B). On the other hand, phosphorylations in
347 the BCR-CK2 α -NF- κ B p65 pathway, including BCR at Y177, CK2 α at Y360, and NF- κ B p65 at
348 S529, were diminished in TMEM147 knockdown cells (Fig. 4C-4E). Furthermore, TMEM147
349 molecules were associated with endogenous BCR and NF- κ B p65, but not CK2 α (Fig. 4F), and the
350 association of BCR with NF- κ B p65 was compromised in TMEM147 knockdown cells (Fig. 4G).
351 These results suggested that TMEM147 acts as a scaffold protein to bind BCR with NF- κ B p65 and
352 is required for the downstream phosphorylation events mediated by the BCR-CK2 α -NF- κ B p65
353 pathway¹⁴.
354

355 **Antibody against TMEM147 suppressed the development of RA models**

356 We then established an antibody against an extracellular domain of TMEM147 molecules (Fig. S8A).
357 Anti-TMEM147 antibody suppressed the activation induced by IL-6 and IL-17A stimulation in
358 ATDC5 cells (Fig. 5A-5C). Notably, joint injections of anti-TMEM147 antibody suppressed
359 cytokine-induced arthritis (Fig. 5D). Furthermore, anti-TMEM147 antibody injections suppressed
360 synovial hyperplasia, cartilage degeneration, and the activation of NF- κ B and STAT3 as well as IL-6
361 mRNA expression in the joints (Fig. 5E-5G, S8B and S8C). Similar results were also obtained in a
362 CIA model (Fig. S9). We were able to observe soft tissue swelling but no bone destruction in both
363 models over a short observation period (Fig. S3J and S3K). Overall, these data strongly suggested
364 that blockade of TMEM147-mediated signaling by an antibody has therapeutic value for RA
365 models..
366

367

DISCUSSION

368

369 During RA development, along with immune cells, including lymphocytes, monocytes, neutrophils,
 370 etc., non-immune cells, including chondrocytes and fibroblasts, in the affected joints are involved in
 371 the pathogenesis. Chondrocytes express MMPs and ADAMTS families in the joints of RA patients,
 372 followed by the degradation of the cartilage^{4 5 30}. However, clinical trials on MMP inhibitors have
 373 failed^{30 31}. In addition, many biological products including antibody drugs have been used for RA
 374 therapy, but a cure remains elusive². These facts suggest more study on the pathological mechanism
 375 is needed.

375

376

377 We found an enhanced production of cartilage-degrading enzymes including MMP13 and
 378 ADAMTS5 in chondrocytes (Fig. S1). Importantly, the conditional deletion of STAT3 in
 379 chondrocytes that abrogates the co-activation of NF-κB and STAT3 significantly suppressed the
 380 clinical symptoms of RA models *in vivo* (Fig. 1E, S3D, S3E, S3H, S3I and S4). These results
 381 indicated that chondrocytes contribute to the inflammation development in RA, most likely by
 382 expressing soluble factors, which may affect both immune cells and non-immune cells. We also
 383 demonstrated that TMEM147 molecules in chondrocytes are critical for the RA development through
 384 NF-κB activation, suggesting a new candidate therapeutic target for RA. Notably, we found the
 385 activation of NF-κB and STAT3 and the enhanced expression of TMEM147 molecules in
 386 osteoarthritis (OA) model mice and OA patients (Fig. S10-S12). Therefore, it is reasonable that the
 387 same TMEM147 pathway might play a role in the inflammation development both in RA and OA. In
 388 addition, other inflammatory joint diseases, such as psoriatic arthritis or gouty arthritis, may develop
 389 through similar mechanisms.

389

390

391 We have reported that many regulatory and target molecules of the inflammation amplifier could be
 392 new markers and therapeutic targets of RA^{8 20}. In this study, we defined TMEM147 as a novel key
 393 molecule for the inflammation amplifier in chondrocytes. It is reported that TMEM147 functions as a
 394 binding partner with membrane proteins. Rosemond et al. reported that TMEM147 binds to M3
 395 muscarinic acetylcholine receptor and acts as a negative regulator of the receptor function on ## cells
 396³². Another study suggested that TMEM147 molecules are associated with the Nicalin-NOMO
 397 complex on ## cells¹⁵. In addition, TMEM147 localizes on the cell surface and is a binding partner
 398 of Haemonchus contortus galectin on goat peripheral blood mononuclear cells {Li, 2016 #45}, and
 399 the membrane-based split-ubiquitin yeast two-hybrid system identified CLN8 and glucagon-like
 400 peptide 1 receptor as binding partners of TMEM147 {Passantino, 2013 #25; Huang, 2013 #26}.
 401 However, it is not known whether TMEM147 is directly involved in the NF-κB pathway. There are
 402 many phosphorylation sites in NF-κB molecules (12). For example, the phosphorylation of p65 S276
 403 is important for DNA binding (13), the phosphorylation of p65 S536 promotes binding with TFIID
 404 (14), and the phosphorylation of p65 S468 is important for p65 transactivation activity (15). We
 405 found that the BCR-CK2α complex together with TMEM147 phosphorylates p65 S529 to establish a
 406 binding site for the acetyltransferase p300 at the promoter region of NF-κB target genes, thus
 407 inducing NF-κB-specific transcription by acetylating NF-κB p65 at K310 and histone H3 at K27
 408 (14)(Fig. 4F and 4G). Thus, we show a new role for TMEM147 as a scaffold protein in NF-κB
 409 signaling (Fig. 6).

409

410

411

412

413

414

415

416 We also found that an antibody directed at an extracellular domain of TMEM147 as well as
 417 TMEM147 deficiency by shRNA-lentivirus suppressed IL-6 production *in vitro* and RA-like arthritis
 418 development *in vivo* (Fig. 3, 5, S8 and S9). These results suggest that TMEM147-mediated NF-κB
 419 activation could be a therapeutic target of RA. We hypothesize that antibody binding to the
 420 extracellular region of TMEM147 may prevent efficient formation of the BCR-CK2α-NF-κB p65
 421 complex intracellularly by sequestering TMEM147 from the cytokine receptor signaling components
 422 in chondrocytes, although we do not exclude the possibility that TMEM147 suppression in other cell

416 types including fibroblasts by the same antibody treatment might also contribute to the *in vivo*
417 effects observed in this study. The precise mechanism of action requires further investigation.
418

419 In summary, our results highlighted the significant contribution of chondrocytes to the development
420 of RA via TMEM147-mediated activation of the inflammation amplifier. This new function of
421 TMEM147 may offer a novel therapeutic strategy for NF- κ B-mediated inflammation in degenerative
422 joint diseases such as RA.

423 **Acknowledgments**

424 We appreciate the excellent technical assistance provided by Ms. C. Nakayama and Ms. M. Ezawa,
425 Mr. Takuto Ohki, and thank Ms. S. Fukumoto (Hokkaido University, Sapporo, Japan) for her
426 excellence assistance. We thank Dr. P. Karagiannis (CiRA, Kyoto University, Kyoto, Japan) for
427 carefully reading the manuscript. This work was supported by JSPS KAKENHI (D. K., Y. A., and M.
428 M.), the Joint Usage/Research Center Institute for Genetic Medicine, Hokkaido University (M. M.),
429 and Takeda Science Foundation (M. M.),

430

431 **Author contribution**

432 All authors were involved in drafting the article or revising it critically for important intellectual
433 content, and all authors approved the final version to be published.

434 **Study conception and design:** Iwasaki and Murakami.

435 **Manuscript writing:** Ota, Kamimura, and Murakami.

436 **Acquisition of data:** Ota, Tanaka, Jiang, and Kamimura.

437 **Analysis and interpretation of data:** Ota, Tanaka, Nakagawa, Jiang, Arima, Kamimura, Onodera,
438 Iwasaki and Murakami.

439

440 **Figure legends**
 441 **Figure 1. Enhanced activation of the inflammation amplifier in chondrocytes is important for**
 442 **RA development in mice.**

443 (A and B) Murine primary chondrocytes were stimulated with human IL-6 plus sIL-6R α and/or
 444 IL-17A or TNF- α , and mRNA (A) or protein (B) levels of IL-6 were measured. IL-6 production was
 445 measured by ELISA specific for mouse IL-6, which was not cross-reactive with human IL-6 added to
 446 the culture. Dot plots in (B) show cell growth measured by the MTT assay (right axis).
 447 (C) Safranin-O staining and immunohistochemistry using anti-phospho-NF- κ B p65 and
 448 phospho-STAT3 antibodies of the ankle in spontaneous RA model mice are shown (n = 5 per group).
 449 Scale bars: 100 μ m.

450 (**D**) The percentage of Safranin-O (+) in the cartilage areas of (C).
 451 (E) The percentages of cells showing phosphorylated NF- κ B p65 and phosphorylated STAT3 in the
 452 cartilage areas. At least 200 cells were counted in each mouse.
 453 (F) The cytokine-induced arthritis model was induced in F759 mice or Col2Cre+ STAT3 flox/flox
 454 mice by ankle joint injections of IL-6 and IL-17A on days 0-2. Clinical scores of the arthritis are
 455 shown (n = 4-8 per group).

456 Data represent the mean \pm S.D. (A, B, D and E) or S.E.M. (F). * p < 0.05, ** p < 0.01 and *** p <
 457 0.001. In vitro experiments were performed at least three times.

458 **Figure 2. Enhanced activation of the inflammation amplifier is observed in chondrocytes from**
 459 **RA patients.**

460 (A) Human primary chondrocytes were stimulated with IL-6 plus sIL-6R α and/or IL-17A or TNF- α ,
 461 and IL-6 mRNA expression was measured.
 462 (B) Safranin-O staining and immunohistochemistry using anti-phospho-NF- κ B p65 and phospho-
 463 -STAT3 antibodies of knee joints in patients with polydactyly (control) or RA are shown (n = 5-6 per
 464 group). Scale bars: 100 μ m.
 465 (C) The percentage of cells showing phosphorylated NF- κ B p65 and phosphorylated STAT3 in the
 466 cartilage areas. At least 200 cells were counted in each patient.
 467 Data represent the mean \pm S.D. * p < 0.05 and *** p < 0.001. In vitro experiments were performed at
 468 least three times.

469 **Figure 3. TMEM147 controls the NF- κ B signaling pathway.**
 470

471 (A and B) Safranin-O staining and immunohistochemistry using anti-TMEM147 antibody or control
 472 IgG of the ankle joints from spontaneous RA models (A) or from RA patients (B) are shown (n = 5-6
 473 per group). Scale bars: 100 μ m.
 474 (C and D) TMEM147-deficient ATDC5 cells were established by using a lentivirus that encoded
 475 shRNA specific for TMEM147. The knockdown efficiency of mRNA (C) and protein (D) levels is
 476 shown.
 477 (E and F) Control (Mock) and TMEM147-deficient ATDC5 (sh) cells were stimulated with or
 478 without human IL-6 plus sIL-6R α and IL-17A, and mRNA (E) or protein (F) levels of IL-6 were
 479 measured. IL-6 production was measured by ELISA specific for mouse IL-6, which was not
 480 cross-reactive with human IL-6 added to the culture. Dot plots in (F) show cell growth measured by
 481 the MTT assay (right axis).
 482 (G and H) Control (Mock) and TMEM147-overexpressing HEK293T cells were stimulated with or
 483 without TNF- α . Reporter activities of NF- κ B (G) and IL-6 (H) promoters are shown.
 484 (I) Control (Mock) and TMEM147-deficient ATDC5 (sh) cells were stimulated with human IL-6
 485 plus sIL-6R α and IL-17A, and mRNA levels of CCL20, CXCL1 and STAT3 were measured.
 486 (J) TMEM147-deficient human primary chondrocytes were established by using TMEM147-specific
 487 siRNA. The knockdown efficiency of TMEM147 at the mRNA level in the presence or absence of

489 human IL-6 plus sIL-6R α and TNF- α stimulation is shown.
 490 (K) Control (si-Control) and TMEM147-deficient (si) human primary chondrocytes were stimulated
 491 with or without human IL-6 plus sIL-6R α and TNF- α . IL-6 mRNA levels were measured.
 492 (L) Control (si-Control) and TMEM147-deficient (si) human primary chondrocytes were stimulated
 493 with human IL-6 plus sIL-6R α and TNF- α , and the mRNA levels of CCL2, CXCL1 and STAT3
 494 were measured.
 495 (M) The cytokine-induced arthritis model that received ankle injections of lentivirus carrying
 496 non-target control shRNA sequence (Mock), shRNA specific to TMEM147 (sh TMEM147) or
 497 NF- κ B p65 (sh p65, a positive control) before IL-6 and IL-17A stimulation. Clinical scores of the
 498 arthritis are shown (n = 4-8 per group).
 499 Data represent the mean \pm S.D. (C, E-L) or S.E.M. (M). * p < 0.05, ** p < 0.01 and *** p < 0.001. In
 500 vitro experiments were performed at least three times.

501
502**503 Figure 4. TMEM147 binds BCR and NF- κ B p65.**

504 (A) Control (Mock) and TMEM147-deficient ATDC5 (sh) cells were stimulated with or without
 505 TNF- α at the indicated time points. The phosphorylation of NF- κ B p65 at S536 and I κ B α was
 506 assessed by western blotting.
 507 (B) Control (Mock) and TMEM147-deficient ATDC5 (sh) cells were stimulated with or without
 508 TNF- α at the indicated time points. The nuclear translocation of NF- κ B p65 was examined by
 509 confocal microscopy. The cellular localization of NF- κ B p65 as a percentage is shown (right). At
 510 least 200 cells were counted in each group. Scale bars: 20 μ m.
 511 (C and D) Control (Mock) and TMEM147-deficient ATDC5 (sh) cells were stimulated with or
 512 without TNF- α at the indicated time points. The phosphorylation of BCR at Y177 and CK2 α at Y360
 513 was assessed by western blotting.
 514 (E) Control (Mock) and TMEM147-deficient ATDC5 (sh) cells were stimulated with or without
 515 TNF- α for 5 min, then immunoprecipitated with anti-NF- κ B p65 to assess the phosphorylation of
 516 NF- κ B p65 at S529.
 517 (F) Flag-tagged TMEM147 was overexpressed in HEK293T cells. Following immunoprecipitation
 518 (IP) with anti-Flag beads, endogenous NF- κ B p65, BCR and CK2 α , as well as overexpressed
 519 TMEM147 were detected by immunoblotting (IB).
 520 (G) Control (Mock) and TMEM147-deficient ATDC5 (sh) cells were stimulated with TNF- α for 5
 521 min, then immunoprecipitated (IP) with anti-NF- κ B p65. Finally, the samples were immunoblotted
 522 (IB) with anti-BCR or anti-NF- κ B p65.
 523 Experiments were performed at least three times; representative data are shown.
 524

525 Figure 5. Anti-TMEM147 antibody suppresses the development of RA-like arthritis.

526 (A, B and C) ATDC5 cells were stimulated with human IL-6 plus sIL-6R α and IL-17A in the
 527 presence or absence of anti-TMEM147 antibody. The mRNA levels of IL-6 (A) and CXCL1 (B), and
 528 protein levels of IL-6 (C) were measured. IL-6 production was measured by ELISA specific for
 529 mouse IL-6, which was not cross-reactive with human IL-6 added to the culture. Dot plots in (C)
 530 show cell growth measured by the MTT assay (right axis).
 531 (D) The cytokine-induced arthritis model was prepared. Anti-TMEM147 antibody was injected into
 532 the ankle joints together with IL-6 and IL-17A. Clinical scores of the arthritis are shown (n = 5 per
 533 group).
 534 (E) HE and Safranin-O staining of the ankle joints from the cytokine-induced arthritis model are
 535 shown. Scale bars: 100 μ m.
 536 (F) The percentage of Safranin-O (+) in the cartilage areas of (E).

537 **(G) The mRNA level of IL-6 in the ankles of (D).**

538 Data represent the mean \pm S.D. (A-C, F and G) or S.E.M. (D). * $p < 0.05$, ** $p < 0.01$ and *** $p <$
539 0.001. In vitro and ex vivo experiments were performed at least three times.

540

541 **Figure 6. Graphical abstract.**

542 Activation of NF- κ B p65 and STAT3 (the inflammation amplifier) in the chondrocytes of RA
543 patients contributes to the collapse of articular homeostasis. TMEM147 acts as a scaffolding protein
544 for binding NF- κ B p65 with BCR, which facilitates the phosphorylation of NF- κ B p65 at S529 and
545 BCR at Y177 to activate the NF- κ B signaling pathway. Target genes of the inflammation amplifier,
546 such as cytokines, chemokines, growth factors, and the MMP and ADAMTS families contribute to
547 the arthritis development.

548 **Supplementary Figure 1. The inflammation amplifier synergistically enhances the expression
549 of MMP13 and of ADAMTS5.**

550 (A and B) Murine primary chondrocytes were stimulated with human IL-6 plus sIL-6R α and/or
551 TNF- α , and the mRNA levels of MMP13 (A) and ADAMTS5 (B) were measured.

552 Data represent the mean \pm S.D. * p < 0.05 and ** p < 0.01. Experiments were performed at least
553 three times.

554 **Supplementary Figure 2. The inflammation amplifier exists in ATDC5.**

556 (A and B) ATDC5 cells were stimulated with human IL-6 plus sIL-6R α and/or IL-17A or TNF- α ,
557 and the mRNA (A) or protein (B) levels of IL-6 were measured to reveal activation of the
558 inflammation amplifier. IL-6 production was measured by ELISA specific for mouse IL-6, which
559 was not cross-reactive with human IL-6 added to the culture. Dot plots in (B) show cell growth
560 measured by the MTT assay (right axis).

561 Data represent the mean \pm S.D. *** p < 0.001. Experiments were performed at least three times.

562 **Supplementary Figure 3. Computed tomography analysis for soft tissue swelling and bone
563 destruction.**

564 Each ankle specimen was scanned by computed tomography (CT).

565 (A) Wild type C57BL/6 mice (8-weeks old).

566 (B-G) The cytokine-induced arthritis model (cytokine-induced F759 arthritis) was prepared. Control
567 antibody (B) or anti-TMEM147 antibody (C) was injected into the ankle joints together with IL-6
568 and IL-17A. F759 mice (D) or Col2Cre+ STAT3 flox/flox mice (E) were injected with IL-6 and
569 IL-17A into the ankles. F759 that had received ankle injections of a lentivirus carrying non-target
570 control shRNA sequence (F) or shRNA specific to TMEM147 (G) before IL-6 and IL-17A
571 stimulation.

572 (H and I) The spontaneous RA model (12-month old F759 mice) was prepared. F759 mice (H) and
573 Col2Cre+ STAT3 flox/flox mice (I) were inspected.

574 (J and K) The collagen-induced arthritis model in C57BL/6 mice (C57BL/6 CIA) was prepared.
575 Control antibody (J) or anti-TMEM147 antibody (K) was injected into the ankle joints every three
576 days starting 14 days after the first immunization.

577 **Supplementary Figure 4. Inflammation amplifier activation and TMEM147 enhancement are
578 observed in knee chondrocytes of F759 mice.**

579 (A) Safranin-O staining and immunohistochemistry using anti-phospho-NF- κ B p65 and
580 phospho-STAT3 antibodies of the knee in the spontaneous RA model mice are shown (n = 5 per
581 group). Scale bars: 100 μ m.

582 (B) The percentage of Safranin-O (+) in the cartilage areas of (A).

583 (C) The percentage of cells showing phosphorylated NF- κ B p65 and phosphorylated STAT3 in the
584 cartilage areas. At least 200 cells were counted in each mouse.

585 (D) Immunohistochemistry using anti-TMEM147 antibody or control IgG of the knee joints (n = 5
586 per group). Scale bars: 100 μ m.

587 Data represent the mean \pm S.D. (B and C). *** p < 0.001.

588 **Supplementary Figure 5. Inflammation amplifier activation in chondrocytes is important for
589 RA development in mice.**

590 (A) HE staining, Safranin-O staining and immunohistochemistry using anti-phospho-NF- κ B p65 and
591 phospho-STAT3 antibodies of the ankle in the cytokine-induced arthritis model are shown (n = 4-8
592 per group). Scale bars: 100 μ m.

593 (B) The percentage of Safranin-O (+) in the cartilage areas of (A).

597 (C) The percentage of cells showing phosphorylated NF- κ B p65 and phosphorylated STAT3 in the
598 cartilage areas. At least 200 cells were counted in each mouse.

599 (D) Immunohistochemistry using anti-TMEM147 antibody or control IgG of the ankle joints (n = 5
600 per group). Scale bars: 100 μ m.

601 Data represent the mean \pm S.D. (B and C). * p < 0.05, ** p < 0.01 and *** p < 0.001.

602 **Supplementary Figure 6. TMEM147 enhances NF- κ B stimulation.**

604 (A and B) Control (Mock) and TMEM147-overexpressing HEK293T cells were stimulated with or
605 without TNF- α . The mRNA levels of TMEM147 (A) and of IL-6 (B) were measured.

606 Data represent the mean \pm S.D. * p < 0.05 and *** p < 0.001. In vitro experiments were performed at
607 least three times.

608 **Supplementary Figure 7. Inflammation amplifier activation and cartilage degeneration are**

609 **suppressed by TMEM147 knockdown in cytokine-induced arthritis in F759 mice.**

611 The cytokine-induced arthritis model (cytokine-induced F759 arthritis) that received ankle injections
612 of lentivirus carrying non-target control shRNA sequence (Mock) or shRNA specific to TMEM147
613 (sh TMEM147) before IL-6 and IL-17A stimulation.

614 (A) HE staining, Safranin-O staining and immunohistochemistry using anti-phospho-NF- κ B p65,
615 anti-phospho-STAT3 and anti-TMEM147 antibodies of the ankle (n = 4-8 per group). Scale bars: 100
616 μ m.

617 (B) The percentage of Safranin-O (+) in the cartilage areas of (A).

618 (C) The percentage of cells showing phosphorylated NF- κ B p65 and phosphorylated STAT3 in the
619 cartilage areas. At least 200 cells were counted in each mouse.

620 Data represent the mean \pm S.D. (B and C). ** p < 0.01 and *** p < 0.001.

621 **Supplementary Figure 8. Anti-TMEM147 antibody injection suppresses the inflammation**

622 **amplifier activation in cytokine-induced arthritis in F759 mice.**

624 (A) ATDC5 cells were treated with the generated anti-TMEM147 antibody, and TMEM147
625 expression on the cell surface was measured by flow cytometry.

626 (B) Immunohistochemistry using anti-phospho-NF- κ B p65 and phospho-STAT3 antibodies of the
627 ankle in the cytokine-induced arthritis model (cytokine-induced arthritis in F759) (n = 5 per group).
628 Scale bars: 100 μ m.

629 (C) The percentage of cells showing phosphorylated NF- κ B p65 and phosphorylated STAT3 in the
630 cartilage areas. At least 200 cells were counted in each mouse.

631 Data represent the mean \pm S.D. (B and C). ** p < 0.01 and *** p < 0.001. In vitro experiments were
632 performed at least three times.

633 **Supplementary Figure 9. Anti-TMEM147 antibody suppresses the development of**

635 **collagen-induced arthritis in C57BL/6 mice.**

636 (A) The cytokine-induced arthritis model in C57BL/6 mice (C57BL/6 CIA) was prepared. Control
637 antibody or anti-TMEM147 antibody was injected into the ankle joints every three days starting 14
638 days after the first immunization. Clinical scores of the arthritis are shown (n = 20 per group).

639 (B) HE staining and Safranin-O staining of the ankle joints. Scale bars: 100 μ m.

640 (C) The percentage of Safranin-O (+) in the cartilage areas of (C).

641 Data represent the mean \pm S.E.M. (A) or S.D. (C). * p < 0.05, ** p < 0.01 and *** p < 0.001.

643 **Supplementary Figure 10. Inflammation amplifier activation and TMEM147 enhancement are**

644 **observed in ankle chondrocytes of OA model.**

645 (A) Safranin-O staining and immunohistochemistry using anti-phospho-NF- κ B p65 and

646 phospho-STAT3 antibodies of the ankle in the spontaneous OA model mice (n = 5 per group). Scale
647 bars: 100 μ m.

648 **(B)** The percentage of Safranin-O (+) in the cartilage areas of (A).

649 **(C)** The percentage of cells showing phosphorylated NF- κ B p65 and phosphorylated STAT3 in the
650 cartilage areas. At least 200 cells were counted in each mouse.

651 **(D)** Immunohistochemistry using anti-TMEM147 antibody or control IgG of the ankle joints (n = 5
652 per group). Scale bars: 100 μ m.

653 Data represent the mean \pm S.D. (B and C). *** p < 0.001.

654

655 **Supplementary Figure 11. Inflammation amplifier activation and TMEM147 enhancement are
656 observed in knee chondrocytes of an OA model.**

657 **(A)** Safranin-O staining and immunohistochemistry using anti-phospho-NF- κ B p65 and
658 phospho-STAT3 antibodies of the knee in spontaneous OA model mice are shown (n = 5 per group).
659 Scale bars: 100 μ m.

660 **(B)** The percentage of Safranin-O (+) in the cartilage areas of (A).

661 **(C)** The percentages of cell showing phosphorylated NF- κ B p65 and phosphorylated STAT3 in
662 cartilage areas. At least 200 cells were counted in each mouse.

663 **(D)** Immunohistochemistry using anti-TMEM147 antibody or control IgG of knee joints from
664 spontaneous OA models (n = 5 per group). Scale bars: 100 μ m.

665 Data represent the mean \pm S.D. (B and C). * p < 0.05, ** p < 0.01 and *** p < 0.001.

666

667 **Supplementary Figure 12. Enhanced activation of the inflammation amplifier and TMEM147
668 enhancement are observed in chondrocytes from OA patients.**

669 **(A)** Safranin-O staining and immunohistochemistry using anti-phospho-NF- κ B p65 and
670 phospho-STAT3 antibodies of knee joints in patients with polydactyly (control) or OA are shown (n
671 = 5 per group). Scale bars: 100 μ m.

672 **(B)** The percentage of cells showing phosphorylated NF- κ B p65 and phosphorylated STAT3 in
673 cartilage areas. At least 200 cells were counted in each patient.

674 **(C)** Immunohistochemistry using anti-TMEM147 antibody or control IgG of knee joints in patients
675 with polydactyly (control) or OA (n = 5 per group). Scale bars: 100 μ m.

676 Data represent the mean \pm S.D. *** p < 0.001.

677

678

679 **Supplementary Table 1. Information of patients.**

680 Rt, right; Lt, left.

681

682 **Supplementary Table 2. Primer sequences used in this study.**

683

684

References

685

686

- 687 1. Makris EA, Gomoll AH, Malizos KN, et al. Repair and tissue engineering techniques for articular
688 cartilage. *Nat Rev Rheumatol* 2015;11(1):21-34. doi: 10.1038/nrrheum.2014.157
- 689 2. Smolen JS, Aletaha D. Rheumatoid arthritis therapy reappraisal: strategies, opportunities and
690 challenges. *Nat Rev Rheumatol* 2015;11(5):276-89. doi: 10.1038/nrrheum.2015.8 [published
691 Online First: 2015/02/17]
- 692 3. Pap T, Korb-Pap A. Cartilage damage in osteoarthritis and rheumatoid arthritis--two unequal
693 siblings. *Nat Rev Rheumatol* 2015;11(10):606-15. doi: 10.1038/nrrheum.2015.95
- 694 4. Smolen JS, Aletaha D, Redlich K. The pathogenesis of rheumatoid arthritis: new insights from old
695 clinical data? *Nat Rev Rheumatol* 2012;8(4):235-43. doi: 10.1038/nrrheum.2012.23
696 [published Online First: 2012/03/13]
- 697 5. Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis.
698 *Immunol Rev* 2010;233(1):233-55. doi: 10.1111/j.0105-2896.2009.00859.x
- 699 6. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006;444:860-67.
- 700 7. Glass CK, Saijo K, Winner B, et al. Mechanisms underlying inflammation in neurodegeneration.
701 *Cell* 2010;140(6):918-34. doi: 10.1016/j.cell.2010.02.016
- 702 8. Murakami M, Harada M, Kamimura D, et al. Disease-association analysis of an
703 inflammation-related feedback loop. *Cell Rep* 2013;3(3):946-59. doi:
704 10.1016/j.celrep.2013.01.028
- 705 9. Atsumi T, Singh R, Sabharwal L, et al. Inflammation amplifier, a new paradigm in cancer biology.
706 *Cancer Research* 2014;74:8-14. doi: 10.1158/0008-5472.CAN-13-2322
- 707 10. Murakami M, Kamimura D, Hirano T. Pleiotropy and Specificity: Insights from the Interleukin 6
708 Family of Cytokines. *Immunity* 2019;50(4):812-31. doi: 10.1016/j.immuni.2019.03.027
- 709 11. Lee J, Nakagiri T, Kamimura D, et al. IL-6 amplifier activation in epithelial regions of bronchi
710 after allogeneic lung transplantation. *Int Immunol* 2013;25(5):319-32. doi:
711 10.1093/intimm/dxs158
- 712 12. Fujita M, Yamamoto Y, Jiang JJ, et al. NEDD4 Is Involved in Inflammation Development during
713 Keloid Formation. *J Invest Dermatol* 2019;139(2):333-41. doi: 10.1016/j.jid.2018.07.044
714 [published Online First: 2018/10/03]
- 715 13. Weisberg E, Manley PW, Cowan-Jacob SW, et al. Second generation inhibitors of BCR-ABL for
716 the treatment of imatinib-resistant chronic myeloid leukaemia. *Nat Rev Cancer*
717 2007;7(5):345-56. doi: 10.1038/nrc2126
- 718 14. Meng J, Jiang JJ, Atsumi T, et al. Breakpoint Cluster Region-Mediated Inflammation Is
719 Dependent on Casein Kinase II. *J Immunol* 2016;197(8):3111-19. doi:
720 10.4049/jimmunol.1601082
- 721 15. Dettmer U, Kuhn PH, Abou-Ajram C, et al. Transmembrane protein 147 (TMEM147) is a novel
722 component of the Nicalin-NOMO protein complex. *J Biol Chem* 2010;285(34):26174-81.
723 doi: 10.1074/jbc.M110.132548
- 724 16. Atsumi T, Ishihara K, Kamimura D, et al. A point mutation of Tyr-759 in interleukin 6 family
725 cytokine receptor subunit gp130 causes autoimmune arthritis. *J Exp Med* 2002;196(7):979-90.
726 [published Online First: 2002/10/09]
- 727 17. Takeda K, Kaisho T, Yoshida N, et al. Stat3 activation is responsible for IL-6-dependent T cell
728 proliferation through preventing apoptosis: generation and characterization of T cell-specific
729 Stat3-deficient mice. *J Immunol* 1998;161(9):4652-60. [published Online First: 1998/10/30]
- 730 18. Sawa S, Kamimura D, Jin GH, et al. Autoimmune arthritis associated with mutated interleukin
731 (IL)-6 receptor gp130 is driven by STAT3/IL-7-dependent homeostatic proliferation of CD4+
732 T cells. *J Exp Med* 2006;12:1459-70.
- 733 19. Murakami M, Okuyama Y, Ogura H, et al. Local microbleeding facilitates IL-6- and

- 734 IL-17-dependent arthritis in the absence of tissue antigen recognition by activated T cells. *J
735 Exp Med* 2011;208(1):103-14. doi: jem.20100900 [pii]
736 10.1084/jem.20100900 [published Online First: 2011/01/12]
- 737 20. Harada M, Kamimura D, Arima Y, et al. Temporal expression of growth factors triggered by
738 epiregulin regulates inflammation development. *J Immunol* 2015;194(3):1039-46. doi:
739 10.4049/jimmunol.1400562
- 740 21. Atsumi T, Suzuki H, Jiang JJ, et al. Rbm10 regulates inflammation development via alternative
741 splicing of Dnmt3b. *Int Immunol* 2017;29(12):581-91. doi: 10.1093/intimm/dxx067
- 742 22. Okuyama Y, Tanaka Y, Jiang JJ, et al. Bmi1 Regulates IkappaBalphalpha Degradation via Association
743 with the SCF Complex. *J Immunol* 2018;201(8):2264-72. doi: 10.4049/jimmunol.1701223
744 [published Online First: 2018/09/14]
- 745 23. Tanaka Y, Sabharwal L, Ota M, et al. Presenilin 1 Regulates NF-kappaB Activation via
746 Association with Breakpoint Cluster Region and Casein Kinase II. *J Immunol*
747 2018;201(8):2256-63. doi: 10.4049/jimmunol.1701446 [published Online First: 2018/09/12]
- 748 24. Stoop R, van der Kraan PM, Buma P, et al. Type II collagen degradation in spontaneous
749 osteoarthritis in C57Bl/6 and BALB/c mice. *Arthritis Rheum* 1999;42(11):2381-9. doi:
750 10.1002/1529-0131(199911)42:11<2381::AID-ANR17>3.0.CO;2-E
- 751 25. Gosset M, Berenbaum F, Thirion S, et al. Primary culture and phenotyping of murine
752 chondrocytes. *Nat Protoc* 2008;3(8):1253-60. doi: 10.1038/nprot.2008.95
- 753 26. Ehlers M, Grotzinger J, deHon FD, et al. Identification of two novel regions of human IL-6
754 responsible for receptor binding and signal transduction. *J Immunol* 1994;153(4):1744-53.
- 755 27. Ogura H, Murakami M, Okuyama Y, et al. Interleukin-17 Promotes Autoimmunity by Triggering
756 a Positive-Feedback Loop via Interleukin-6 Induction. *Immunity* 2008;29:628-36. doi:
757 10.1016/j.jimmuni.2008.07.018
- 758 28. Lee J, Nakagiri T, Oto T, et al. IL-6 amplifier, NF-kappaB-triggered positive feedback for IL-6
759 signaling, in grafts is involved in allogeneic rejection responses. *J Immunol*
760 2012;189(4):1928-36. doi: jimmunol.1103613 [pii]
761 10.4049/jimmunol.1103613 [published Online First: 2012/07/17]
- 762 29. Narimatsu M, Maeda H, Itoh S, et al. Tissue-specific autoregulation of the stat3 gene and its role
763 in interleukin-6-induced survival signals in T cells. *Mol Cell Biol* 2001;21(19):6615-25.
764 [published Online First: 2001/09/05]
- 765 30. Ghoreschi K, Laurence A, Yang XP, et al. Generation of pathogenic T(H)17 cells in the absence
766 of TGF-beta signalling. *Nature* 2010;467(7318):967-71. doi: nature09447 [pii]
767 10.1038/nature09447 [published Online First: 2010/10/22]
- 768 31. Krzeski P, Buckland-Wright C, Balint G, et al. Development of musculoskeletal toxicity without
769 clear benefit after administration of PG-116800, a matrix metalloproteinase inhibitor, to
770 patients with knee osteoarthritis: a randomized, 12-month, double-blind, placebo-controlled
771 study. *Arthritis Res Ther* 2007;9(5):R109. doi: 10.1186/ar2315
- 772 32. Rosemond E, Rossi M, McMillin SM, et al. Regulation of M(3) muscarinic receptor expression
773 and function by transmembrane protein 147. *Mol Pharmacol* 2011;79(2):251-61. doi:
774 10.1124/mol.110.067363
- 775

Figure 1

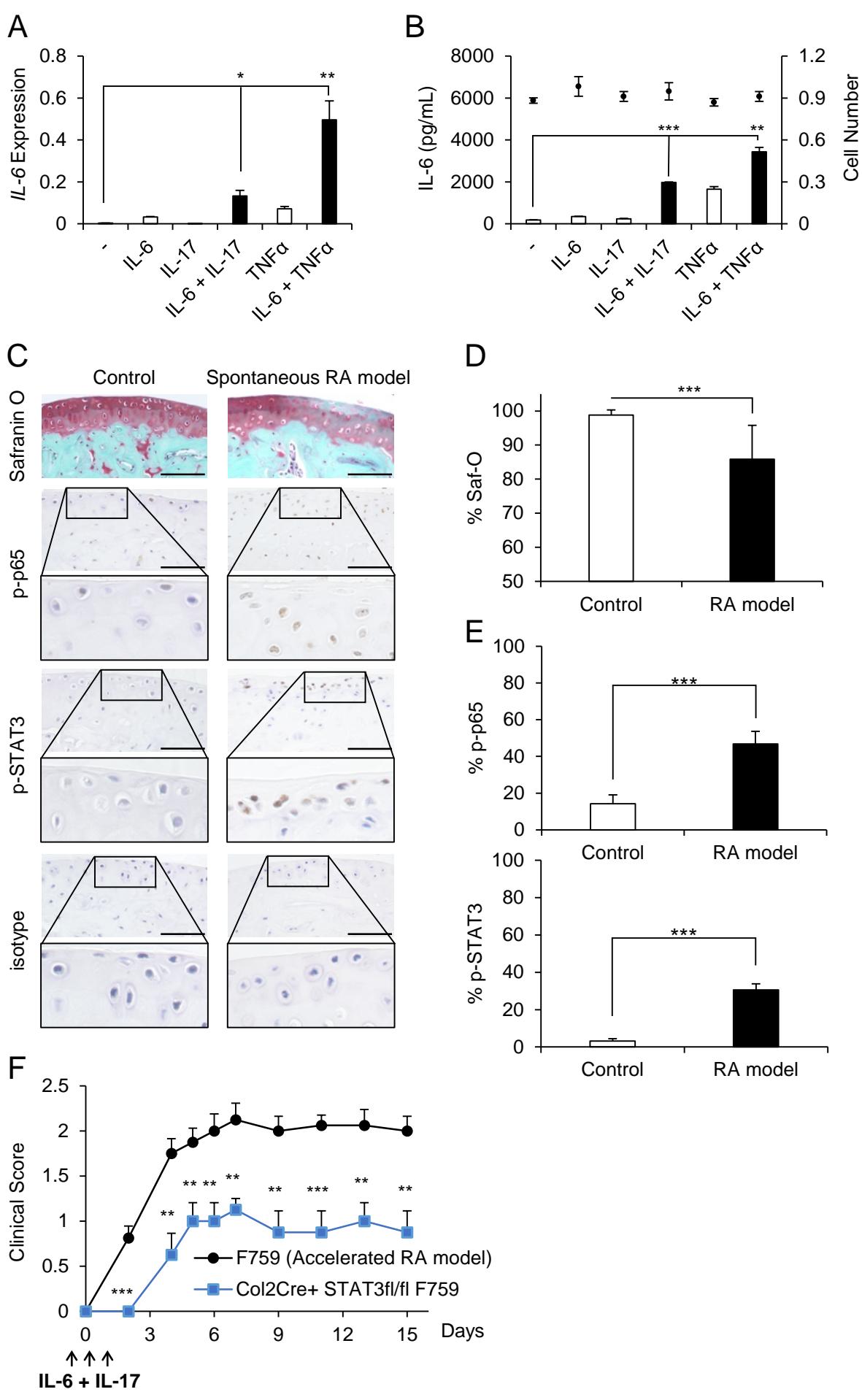


Figure 2

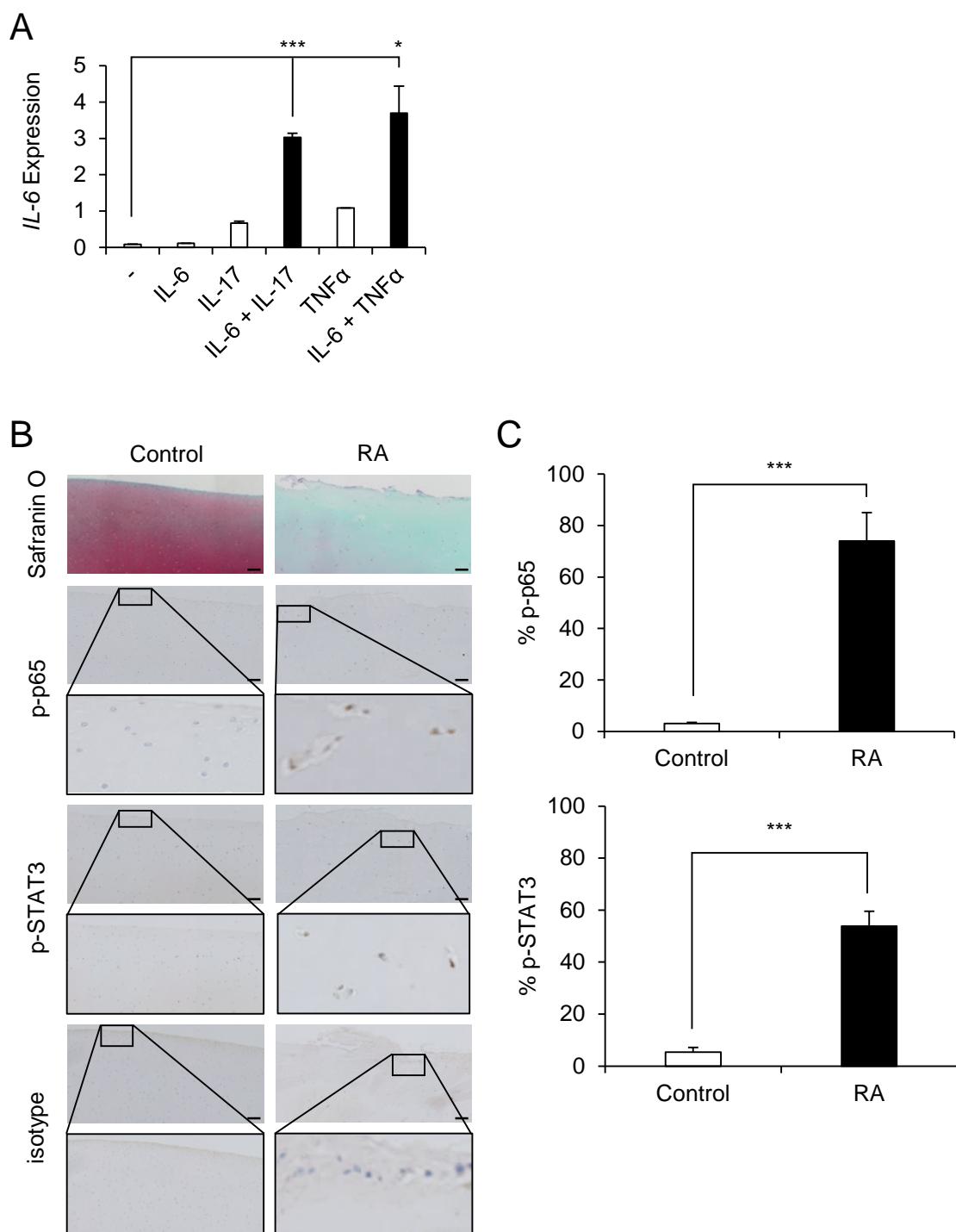


Figure 3

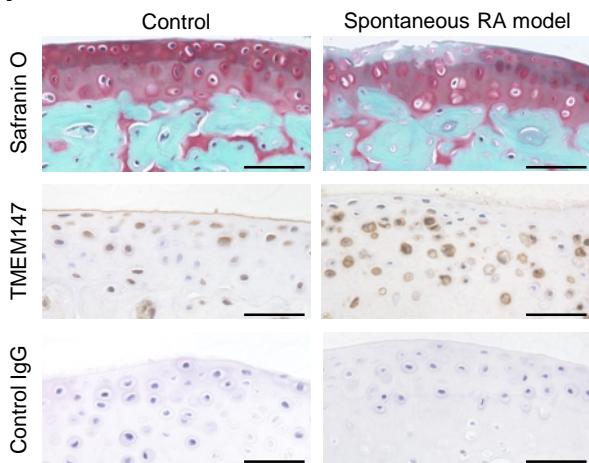
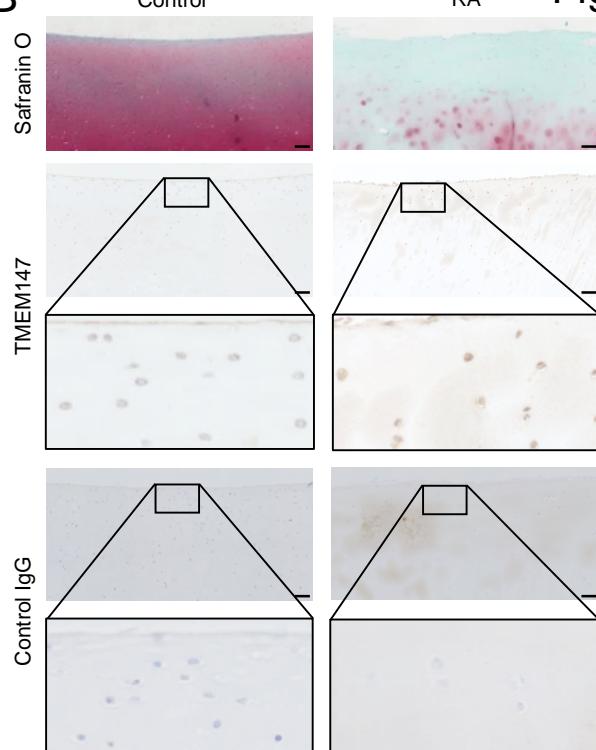
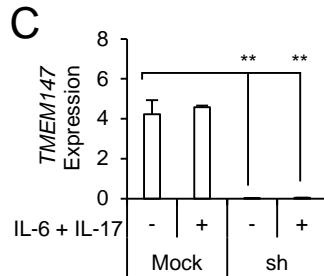
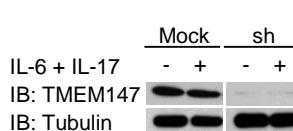
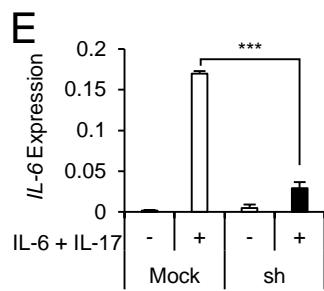
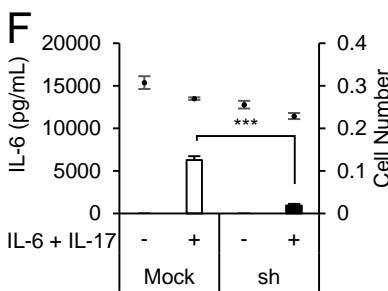
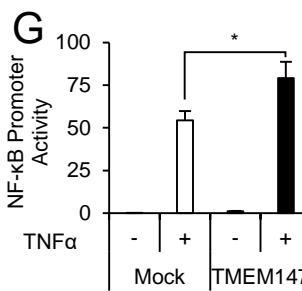
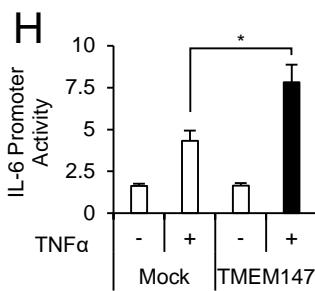
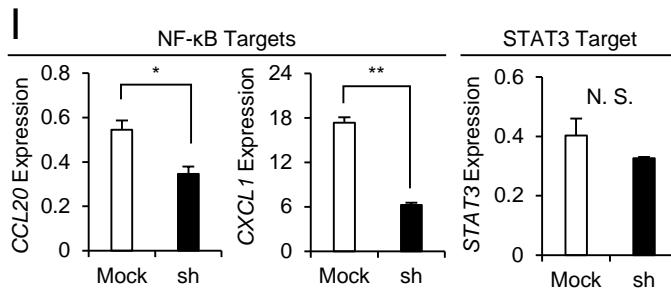
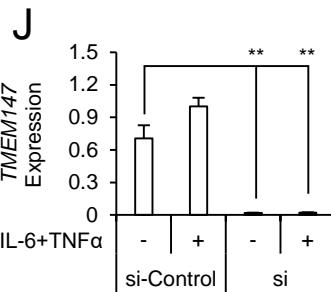
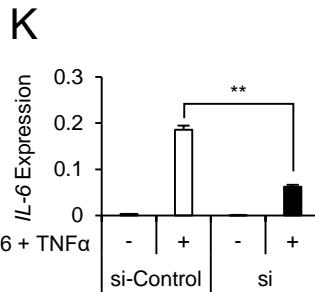
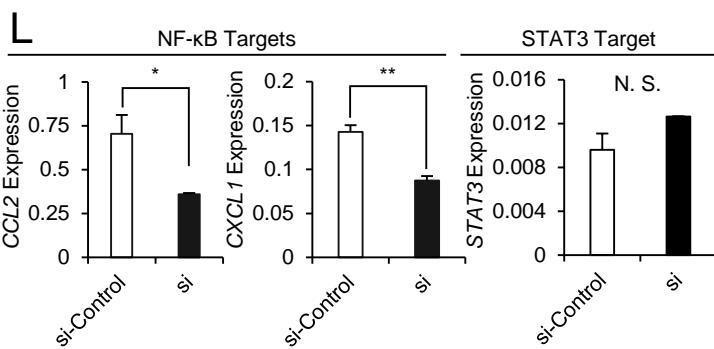
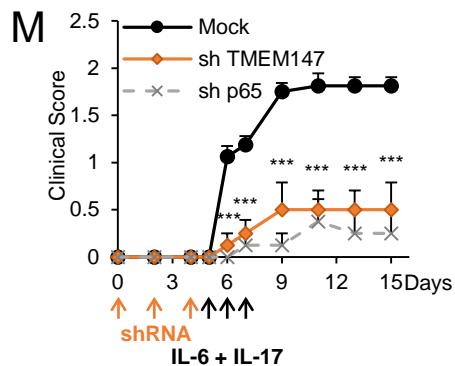
A**B****C****D****E****F****G****H****I****J****K****L****M**

Figure 4

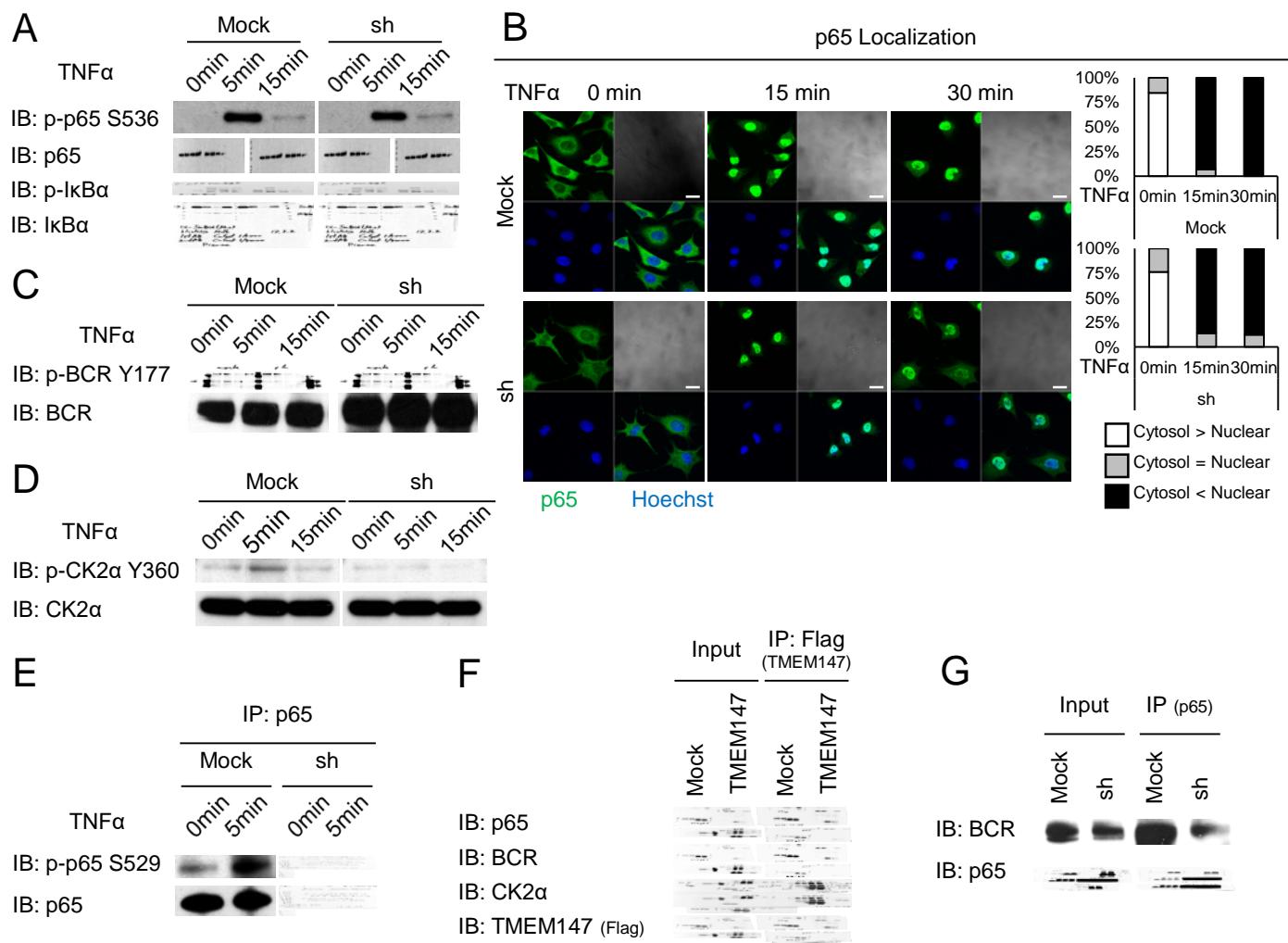
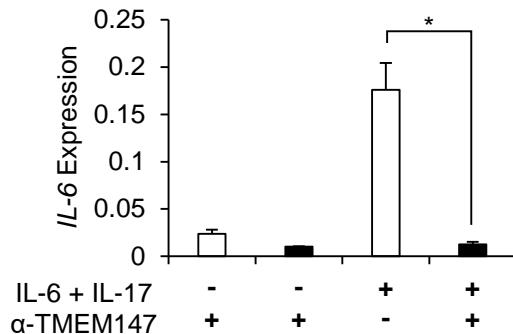
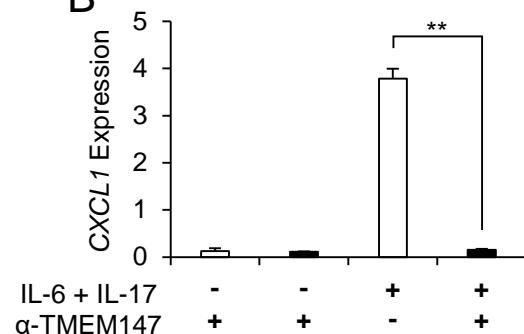


Figure 5

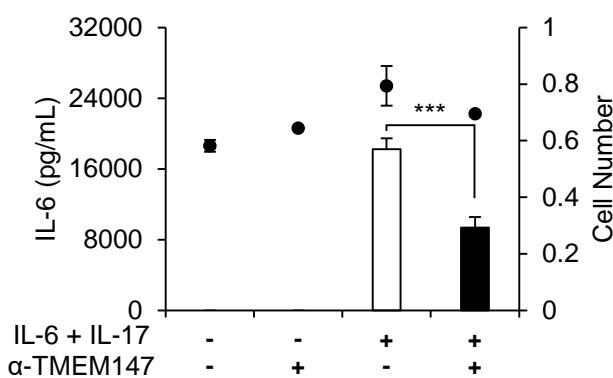
A



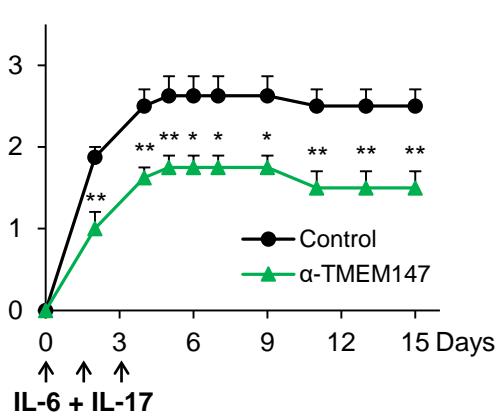
B



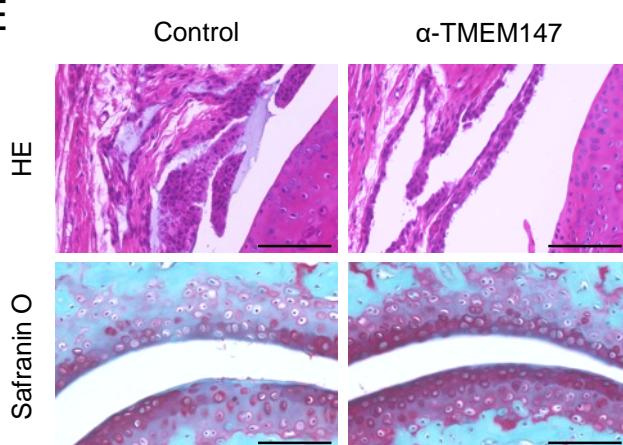
C



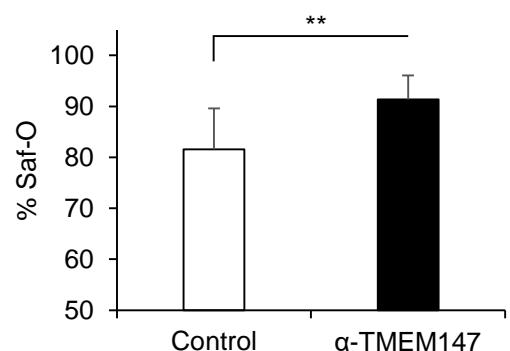
D



E



F



G

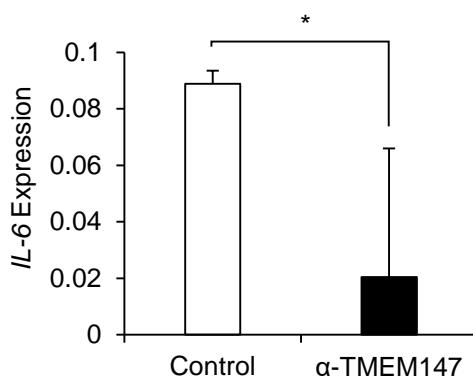
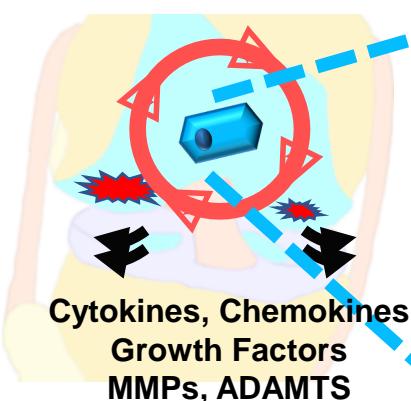


Figure 6

Rheumatoid Arthritis

The Inflammation Amplifier



Chondrocytes

NF-κB Pathway

