MUSCULOSKELETAL PATHOLOGY

Tumor Necrosis Factor Receptor Associated Factor 3 Modulates Cartilage Degradation through Suppression of Interleukin 17 Signaling

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Accepted for publication
April 8, 2020.

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Interleukin 17A (IL-17A) is critical in the pathogenesis of autoimmune diseases through driving inflammatory cascades. However, the role of IL-17 in osteoarthritis (OA) is not well understood. Tumor necrosis factor–receptor–associated factor 3 (TRAF3) is a receptor proximal negative regulator of IL-17 signaling. It remains unclear whether TRAF3 exerts regulatory effects on cartilage degradation and contributes to the pathogenesis of OA. In this study, we found that TRAF3 notably suppressed IL-17–induced NF- κ B and mitogen-activated protein kinase activation and, subsequently, the production of matrix-degrading enzymes. TRAF3 depletion enhanced IL-17 signaling, along with increased matrix-degrading enzyme production. *In vivo*, cartilage destruction caused by surgery-induced OA was alleviated markedly both in *Il17a*–deficient mice and in TRAF3 transgenic mice. In contrast, silencing TRAF3 through adenoviruses worsened cartilage degradation in experimental OA. Moreover, the destructive effect of IL-17 on cartilage was abolished in TRAF3 transgenic mice in an IL-17 intra-articular injection animal model. Similarly, genetic deletion of IL-17 blocked TRAF3 knockdown–mediated promotion of cartilage destruction, suggesting that the protective effect of TRAF3 on cartilage is mediated by its suppression of IL-17 signaling. Collectively, our results suggest that TRAF3 negatively regulates IL-17–mediated cartilage degradation and pathogenesis of OA, and may serve as a potential new therapy target for OA. (*Am J Pathol* 2020, 190: 1701–1712; <https://doi.org/10.1016/j.ajpath.2020.04.016>)

Osteoarthritis (OA) is the most common joint disease characterized by cartilage destruction, subchondral bone sclerosis, and synovial inflammation.¹ These abnormalities have been attributed mainly to an imbalance between the anabolism and catabolism of the articular cartilage, especially an increase in catabolism.² Proinflammatory cytokines, important mediators of OA, such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and IL-6, are widely investigated in articular cartilage.^{3–5} IL-17 is a crucial proinflammatory factor in the pathogenesis of various autoimmune diseases, such as

Supported by National Natural Science Foundation of China grants 81830078 (X.Z.), 81772347 (X.Z.), 81572123 (X.Z.), Science and Technology Commission of Shanghai Municipality grant 19XD1434100 (X.Z.), Shanghai Municipal Education Commission-Gao feng Clinical Medicine Grant Support 20161314 (X.Z.), Shanghai Jiao Tong University International Collaboration Project, and the Shanghai Jiao Tong University–The Chinese University of Hong Kong Joint Research Collaboration Fund (X.Z.).

G.H. and N.Z. contributed equally to this work.

Disclosures: The authors have submitted a patent on TRAF3 modulates cartilage degradation (patent application number 202010350036.8).

experimental autoimmune encephalomyelitis,⁶ collagen-induced arthritis,⁷ and inflammatory bowel disease.⁸ IL-17 is a major inflammation-driving cytokine, exerting its functions through inducing and sustaining the production of inflammatory cytokines, chemokines, and matrix metalloproteinases.⁹ IL-17 also can act synergistically with IL-1 β or TNF- α for further induction of proinflammatory genes.^{10,11} In rheumatoid arthritis, synovial fibroblast IL-17 has been proven important because it induces the production of inflammatory factors IL-6 and IL-8 via the NF- κ B and phosphatidylinositol 3-kinase/Akt pathways.¹² During OA, IL-17 is an upstream factor in the regulation of IL-1 β and TNF- α .^{4,13} In articular cartilage and chondrocytes, IL-17 up-regulates the mRNA expression of several cartilage catabolic factors such as IL-6, nitric oxide, and matrix metalloproteinases (MMPs) through NF- κ B and mitogen-activated protein kinase (MAPK) pathways.^{13–15} IL-17 also is more active in chondrocytes than in fibroblasts during OA pathophysiology.¹⁶ Interestingly, significant increases in circulating T helper 17 cells were observed in patients after anti-TNF- α therapy. Thus, the combined blockade of TNF- α and IL-17 in experimental arthritis was more effective than monotherapy.¹⁷ Therefore, blocking the IL-17 pathway is a promising method for preventing cartilage from degradation in arthritis.

The TNF-associated factors (TRAFs) are a family of intracellular adaptor proteins that mediate downstream signaling of various cell surface receptors and thus control the early stages of cellular defense mechanisms against invading bacterial and viral pathogens. Most members of this family, including TRAF2, TRAF5, and TRAF6, have been studied intensively, however, investigations on TRAF3 remain scarce.¹⁸ The up-regulation of TRAF3 expression in osteoclast precursors can mitigate bone destruction and inflammation-induced bone loss in common bone diseases.¹⁹ Moreover, TRAF3 is a critical factor in controlling the induction of type I interferon²⁰ and the expression of the anti-inflammatory cytokine IL-10.²¹ These findings suggest that TRAF3 plays a complicated role in restoring immunologic homeostasis. Recent reports have indicated that TRAF3 could play a negative role in IL-17-induced NF- κ B and MAPK signaling pathways and that knockdown of TRAF3 greatly enhanced IL-17 signaling in human primary synoviocytes.²² As previously mentioned, IL-17 has a greater influence on chondrocytes than synoviocytes during OA process.¹⁶ The effect of TRAF3 on chondrocytes and protecting cartilage from degradation has yet to be investigated further.

In this study, we showed that overexpression of TRAF3 significantly blocked IL-17-induced NF- κ B and MAPK pathways in chondrocytes and consequently reduced the production of IL-6 and MMPs. Conversely, knocking down TRAF3 resulted in increased MMP-13 production induced by IL-17. *In vivo*, loss of IL-17 markedly alleviated cartilage destruction caused by surgery-induced OA and age-related spontaneous OA. TRAF3 transgenic mice showed less cartilage destruction in different arthritic animal models compared

with littermate control. Furthermore, silencing TRAF3 by using adenoviruses markedly worsened cartilage degradation in experimental OA, which partially was rescued by IL-17 deficiency. Collectively, TRAF3 is likely to be a potential therapeutic target for ameliorating arthritis cartilage degradation.

Materials and Methods

Animals

TRAF3 transgenic mice and *Il17a*-deficient (*Il17a*^{-/-}) mice were a kind gift from Professor Youcun Qian from the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). The generation of TRAF3 transgenic mice was described in a previous study.²² The TRAF3 transgenic mice (T3TG) and *Il17a*^{-/-} mice and littermate [wild-type (WT)] controls at 8 to 12 weeks of age were used for experiments. C57BL/6 mice and Sprague Dawley rats were purchased from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences). All of the animals were maintained under pathogen-free conditions. All of the animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Biomedical Research Ethics Committee of the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences).

IL-17 Intra-Articular Injection Model

Mice were anesthetized with 250 mg/kg intraperitoneal tribromoethanol (Sigma, St. Louis, MO). A total volume of 5 μ L containing 10 ng/mL recombinant IL-17 (R&D, McKinley, MN) in phosphate-buffered saline (PBS) was injected through the infrapatellar ligament into the left knee joint space using a 30 G needle (65460-05; Hamilton Co., Bonaduz, Switzerland) as previously described,²³ and the patellar ligament was made visible through a small skin incision. The right knee was injected with 5 μ L PBS as a control group. An intra-articular injection of IL-17 (10 ng/mL in PBS) and PBS into the T3TG mice and littermate controls three consecutive times every 24 hours was performed. Three days after the last injection, T3TG and WT mice were euthanized through CO₂ administration ($n = 10$ per group). The samples of the knee joints were collected and fixed in 4% paraformaldehyde.

Destabilization of the Medial Meniscus Model and Osteoarthritis Research Society International Scoring

Mice destabilization of the medial meniscus (DMM) was induced by transection of the anterior attachment of the medial meniscus to the tibia plateau.^{24,25} Knees then were prepared for aseptic surgery. A longitudinal incision medial to the patellar ligament was made, the joint capsule was opened, and the meniscotibial ligament, which anchors the medial meniscus to the tibial plateau, was identified. No

further manipulation was performed on a subset of animals considered as the sham group. In the experimental group, the medial meniscotibial ligament was transected, resulting in DMM. In both sham and DMM animals, the joint capsule and subcutaneous layer were sutured and closed separately. Six weeks after DMM, T3TG and WT mice were euthanized through CO₂ administration. For adenovirus injection, control viruses (Ad-C) or adenoviruses containing TRAF3 short hairpin RNA plasmid (Ad-sh-*Traf3*) viruses were injected intra-articularly once a week 1 week after DMM surgery for 5 weeks. The knee joints were collected and fixed in 10% buffered formalin overnight at 4°C, followed by 2-week decalcification in 14% EDTA. Joints then were embedded in paraffin and sectioned at 5-μm thickness. Histologic slides were prepared and sections were stained using safranin-O (S2255; Sigma) and fast green (F7252; Sigma), and then graded for OA severity by four independent graders using the Osteoarthritis Research Society International scoring system.^{26,27} Three sections (medial, central, and lateral) from each joint were stained and evaluated. The three sections were graded separately and averaged for each joint.

Rat OA Animal Model Human Cartilage Sample

Rat OA was induced by DMM surgery as described in the previous paragraph. Briefly, the animals were anesthetized, and surgery was performed to transect the medial collateral ligament. The medial meniscus was cut through the full thickness to induce the joint destabilization of the right knee. Sham animals underwent the same surgical procedure without any ligament transection or meniscal tear as the control group. The rats that underwent surgery were euthanized at 1, 2, or 3 months after surgery ($n = 7$ to 10 per group), the samples of the knee joints were collected and fixed in 4% paraformaldehyde. Human OA cartilage was sampled from patients who underwent a total joint replacement because of primary OA of the knee ($N = 3$). Undamaged areas in the same patient were sampled as the normal cartilage. Ethical approval was obtained from the Medical Ethics Committee of the Shanghai Ninth People's Hospital (Shanghai Jiao Tong University School of Medicine, Shanghai, China), and informed consent was obtained from all participants.

Histology and Immunohistochemistry

Knee joints isolated from the experimental animals were fixed overnight with 4% paraformaldehyde decalcified by 12.5% EDTA in PBS (pH = 7.0) and then embedded in paraffin. Tissue sections (5 μm) were deparaffinized in xylene, serially rehydrated in ethanol, and washed with PBS. Sections were stained with safranin O/fast green to identify proteoglycan loss. Safranin O staining was quantified according to density using ImageJ software version 1.52t (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>). For immunohistochemistry, sections in 10 mmol/L sodium citrate buffer (pH 6.0) were heated in a

microwave oven at 95°C for 10 minutes. Slides were cooled for 30 minutes at room temperature after antigen unmasking. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and was rinsed several times with PBS. After blocking nonspecific protein binding with 5% bovine serum albumin in PBS for 30 minutes at room temperature, sections were incubated overnight at 4°C with primary antibodies against TRAF3 (ab217033; Abcam, Cambridge, UK) or MMP-13 (ab219620; Abcam). The slides were rinsed with PBS and then incubated with secondary antibody (Cell Signaling Technology, Danvers, MA) in accordance with the manufacturer's instructions. The sections then were counterstained with Mayer's hematoxylin (Sigma). After being washed, the slides were stained with 3,3'-diaminobenzidine tetrahydrochloride (EnVision detection kit, peroxidase/diaminobenzidine, rabbit/mouse; Dako Cytomation, Santa Clara, CA). Staining with normal IgG and staining without primary antibodies also was performed as a negative control. The immunohistochemistry results were quantified through calculating the percentage of positive cells using ImageJ software.

Cell Culture

Rat Chondrocytes Isolation and Culture

Primary rat chondrocytes were isolated from the rats' knee cartilage, after digestion by type II collagenase for 5 hours at 37°C, filtered using a 70-μm filter (BD Corning, Franklin Lakes, NJ), and then cultured in a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Thermo Fisher Scientific, Waltham, MA) with 10% fetal bovine serum (Thermo Fisher Scientific), 100 U/mL penicillin G, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B under a humidified atmosphere of 5% CO₂ in air at 37°C.

Chondrogenic Cell Line ATDC5

The murine chondrogenic ATDC5 cell line was obtained from RIKEN Cell Bank (Tsukuba, Japan). Cultures of undifferentiated ATDC5 cells were maintained in a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium and supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 2.5/mL amphotericin B.

Chondrosarcoma Cell Line SW1353

The human chondrosarcoma cell line SW1353 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences. The cells were cultured similarly in a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium.

Transient Transfections Gene Expression

Flag-tagged *TRAF3* was cloned into a retrovirus-mediated pMSCV-IRES-GFP vector (kindly provided by Youcun Qian's laboratory, Institute of Health Science, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) and a pcDNA 3.0 plasmid. ATDC5

Figure 1 Interleukin (IL)-17 activates NF- κ B and mitogen activated protein kinase (MAPK) signaling and up-regulates catabolic factors in chondrocytes. **A–C:** The mRNA levels of catabolic genes in primary rat chondrocytes, the murine ATDC5 cell line, and the human chondrosarcoma SW1353 cell line stimulated with IL-17 (24 hours, 10 ng/mL). **D–F:** Immunoblotting of the main molecules involved in the NF- κ B and MAPK signaling pathways in IL-17-stimulated primary chondrocytes, the ATDC5 cell line, and the SW1353 cell line. **G–I:** Quantifications of the immunoblotting results. Data are expressed as means $\pm$ SEM. $n \geq 3$ independent experiments (**A–I**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control. Adamts, a disintegrin and metalloproteinase with thrombospondin motifs; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinase; min, minutes; MMP, matrix metalloproteinase; p-ERK, phospho-ERK; p-JNK, phospho-JNK.

Figure 2 Tumor necrosis factor-receptor-associated factor 3 (Traf3) negatively regulates interleukin (IL)-17-mediated signaling and the induction of matrix-degrading enzymes in chondrocytes. **A** and **B**: Primary rat chondrocytes (**A**) or murine ATDC5 cells (**B**) transfected with plasmids for empty vector (EV) or M2 (Flag)-tagged TRAF3 were left untreated or treated with IL-17 (10 ng/mL) for 15 or 30 minutes. Whole-cell lysates were immunoblotted with anti-p65, anti-p-p65, anti-p-JNK, anti-p38, anti-p-p38, anti-ERK, anti-p-ERK, anti-TRAF3, or anti-β-actin. **C** and **D**: *Il6*, *Mmp13*, a disintegrin and metalloproteinase with thrombospondin motifs *Adamts4*, and *Adamts5* mRNA expression induced by IL-17 (10 ng/mL) in primary chondrocytes (**C**) or ATDC5 cells infected with adenoviruses containing empty vector (Ad-C) or adenoviruses containing the entire CDS of TRAF3 (Ad-Traf3) (**D**). **E**: Western blot analysis of catabolic factors (Mmp13 and COX-2) in primary chondrocytes infected with Ad-C or Ad-Traf3. **F**: Immunoblotting of catabolic factors (Mmp13 and COX-2) in primary chondrocytes infected with Ad-C or adenoviruses containing TRAF3 shRNA plasmid (Ad-sh-Traf3). **G–J**: Quantification of the immunoblotting results. Data are expressed as means ± SEM. $n \geq 3$ independent experiments (**A–J**). * $P < 0.05$, ** $P < 0.01$ versus control. COX-2, cyclooxygenase-2; Ctrl, control; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; MMP, matrix metalloproteinase; p-ERK, phospho-ERK; p-JNK, phospho-JNK.

cells were infected by viral supernatant suspensions obtained from human embryonic kidney (HEK) 293 FT cells transfected with flag-tagged TRAF3 plasmid together with help vectors. SW1353 cells were transfected by pcDNA-TRAF3 and empty pcDNA vectors with the use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

RNA Interference

RNA interference was performed using siGENOME SMART pool siRNA (Dharmacon, Lafayette, CO) targeting rat TRAF3 interacting protein 2 (TRAF3IP2 or Act1) (gene ID: 10758).

Figure 3 Interleukin (IL)-17 deficiency attenuates cartilage matrix degradation in surgery-induced osteoarthritis (OA) and aging-induced spontaneous OA. **A:** Cartilage destruction and Osteoarthritis Research Society International (OARSI) grade in sham-operated and destabilization of the medial meniscus (DMM)-operated wild-type (WT) and *Il17a*-deficient (*Il17a*^{-/-}) mice at the age of 3 months. **B:** Safranin-O staining and OARSI grade in 14-month-old WT and *Il17a*^{-/-} mice. Data are expressed as means $\pm$ SEM. $n = 6$ (A); $n = 8$ (B). $^{**}P < 0.01$ versus control. Scale bars: 100 μ m (A); 50 μ m (B). KO, knockout.

RNA Purification and Quantitative Real-Time PCR

Total RNA was prepared using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. A first-strand cDNA was synthesized from 1 μ g of the total RNA by incubating for 1 hour at 42°C with Superscript III reverse transcriptase (Invitrogen) after oligo deoxythymine (dT) priming. After the reverse-transcription reaction, quantitative real-time PCR was performed with a LightCycler480 system (Roche, Basel, Switzerland) using SYBR1Premix Ex Taq (Takara Bio, Shiga, Japan) in accordance with the manufacturer's instructions. The conditions of real-time PCR were as follows: denaturation at 95°C for 10 seconds, 40 cycles at 95°C for 10 seconds, and 60°C for 30 seconds. The dissociation stage was added to the end of the amplification procedure. No nonspecific amplification was determined by the dissociation curve. All of the amplifications were normalized by β -actin. Data were analyzed using the comparison Ct ($2^{-\Delta\Delta C_t}$) method and expressed as fold change compared with the respective control.

Western Blot

Cells were lysed on ice for 30 minutes in the lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors (10 mg/mL leupeptin, 10 mg/mL pepstatin

A, and 10 mg/mL aprotinin). For Western blot analysis, a 50 μ g protein sample was resolved on 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes (Whatman, Piscataway, NJ). The primary antibodies used were anti-TRAF3 (1:1000; Abcam), MMP-13 (1:200, sc-30073; Santa Cruz Biotechnology, Santa Cruz, CA), cyclooxygenase (COX)-2 (1:500, ab15191; Abcam), glyceraldehyde-3-phosphate dehydrogenase (1:5000, G9545; Sigma), anti-phospho-p65 (1:1000), anti-p65 (1:1000), anti-phospho-p38 MAPK (1:1000), anti-p38 MAPK (1:1000), anti-phospho-p44/42 MAPK (1:2000), anti-p44/42 MAPK (1:1000), anti-phospho-c-Jun N-terminal kinase (JNK) (1:1000), and anti-JNK (1:1000), which all were purchased from Cell Signaling Technology. β -actin was used as a loading control. Horseradish-peroxidase-conjugated secondary antibodies (Cell Signaling Technology) were used at a 1:1000 dilution. The antigen-antibody complexes were made visible using an enhanced chemiluminescence detection system in accordance with the manufacturer's recommendations. Immunoreactive bands were analyzed quantitatively in triplicate by normalizing the band intensities into β -actin on scanned films with the use of ImageJ.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 6 (GraphPad, Durham, NC). All data are

Figure 4 Tumor necrosis factor—receptor—associated factor 3 (TRAF3) transgenic mice (T3TG) show less matrix metalloproteinase 13 (MMP-13) production and cartilage extracellular matrix loss induced by interleukin (IL)-17. **A and B:** Representative images and quantification of immunohistochemistry of MMP-13 in T3TG and littermate control mice after intra-articular injection of IL-17 or phosphate-buffered saline (PBS). **Boxed region** shows MMP-13–positive chondrocytes. **C:** Safranin-O staining of the articular cartilage of T3TG and littermate control after intra-articular injection of IL-17 or PBS. **Boxed region** shows magnified articular cartilage. Data are expressed as means $\pm$ SEM. $n = 6$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 50 μ m (**A** and **C**); 20 μ m (**A** and **C**, higher magnification). IHC, immunohistochemistry; WT, wild-type.

shown as means $\pm$ SD. Statistical differences between two groups were determined by a two-tailed t -test. P values less than 0.05 were considered statistically significant. All experiments were performed on at least 3 individual samples.

Results

IL-17 Induces the Production of Inflammatory Cytokines, MMPs, and Aggrecanase in Chondrocytes

To investigate the role of IL-17 in chondrocytes catabolism, chondrocytes were treated with recombinant IL-17 (10 ng/mL, 24 hours). IL-17 treatment resulted in significant

up-regulation of *IL6*, *MMP13*, a disintegrin and metalloproteinase with thrombospondin motifs-4 (*ADAMTS4*) and *ADAMTS5* at the mRNA level, which play crucial roles in OA cartilage destruction in rat primary chondrocytes (Figure 1A), ATDC5 cells (Figure 1B), as well as SW1353 cells (Figure 1C). Moreover, treatment of IL-17 promptly triggered the phosphorylation of p65, p38, extracellular signal-regulated kinase (ERK), and JNK in rat primary chondrocytes (Figure 1, D and G) and ATDC5 cells (Figure 1, E and H). However, IL-17 only activated the phosphorylation of p65, ERK, and JNK, but not p38 in SW1353 cells (Figure 1, F and I). Altogether, these data suggest that IL-17 up-regulates cartilage catabolic factors through activation of NF- κ B and MAPK signaling.

Figure 5 Increased tumor necrosis factor—receptor—associated factor 3 (Traf3) expression during experimental osteoarthritis (OA) pathogenesis. **A:** Immunohistochemistry of Traf3 expression in rat cartilage obtained from destabilization of the medial meniscus (DMM)-induced experimental OA or sham groups at 1 month, 2 months, and 3 months after surgery. **B:** Fold change of *Traf3* mRNA expression level in cartilage obtained from the rat described in panel A. **C:** Immunohistochemistry of TRAF3 expression in cartilage obtained from OA patients. **Boxed region** shows TRAF3 positive chondrocytes. Data are expressed as means $\pm$ SEM. $n = 8$ (A, 1 month); $n = 6$ (A, 2 months); $n = 9$ (A, 3 months); $n = 3$ (C). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 20 μ m (A, C, higher magnification); 50 μ m (C). IHC, immunohistochemistry.

TRAF3 Tightly Controls IL-17–Induced Expression of Catabolic Genes

To determine the role of TRAF3 in IL-17–mediated signaling, an adenovirus containing the entire coding sequence (CDS) sequence of *TRAF3* (Ad-*Traf3*) was generated. Indeed, Ad-*Traf3* infection effectively increased the TRAF3 expression level in primary chondrocytes (Supplemental Figure S1, A and B). Overexpression of TRAF3 notably suppressed IL-17–induced p65 phosphorylation, p38 phosphorylation, JNK phosphorylation, and ERK phosphorylation, indicating that TRAF3 has general

inhibitory effects on IL-17–mediated pathways (Figure 2A). Moreover, TRAF3 or empty vector plasmids were also transfected into SW1353 cells and IL-17–mediated immediate signaling was checked. Consistently, IL-17–induced p65 phosphorylation, p38 phosphorylation, and JNK phosphorylation, but not ERK phosphorylation, were inhibited by TRAF3 overexpression (Figure 2B), indicating that TRAF3 has general inhibitory effects on IL-17–mediated pathways in chondrocytes. This study then investigated the role of TRAF3 in IL-17–induced expression of cartilage catabolic factors in chondrocytes. Notably, TRAF3 overexpression suppressed

Figure 6 Tumor necrosis factor–receptor–associated factor 3 (TRAF3) controls osteoarthritis (OA) development likely through its suppression of interleukin (IL)-17 signaling. **A:** Cartilage destruction and Osteoarthritis Research Society International (OARSIS) grade in sham-operated or destabilization of the medial meniscus (DMM)-operated wild-type (WT) and TRAF3 transgenic mice (T3TG) mice. **Boxed regions** show the magnified articular cartilage. **B:** Safranin-O staining and OARSIS grade of articular cartilage harvested from sham- or DMM-operated WT or *Il17a*^{-/-} mice injected intra-articularly with adenoviruses containing empty vector (Ad-C) or adenoviruses containing TRAF3 shRNA plasmid (*Ad-sh-Traf3*). Data are expressed as means ± SEM. *n* = 12 (**A**); *n* = 10 (**B**). **P* < 0.05, ***P* < 0.01. Scale bars: 100 μm (**A**); 50 μm (**A**, higher magnification, and **B**). KO, knockout.

IL-17–induced up-regulation of *Il6*, *Mmp13*, and *Adamts4*, but not *Adamts5*, at the mRNA level in both primary chondrocytes and ATDC5 cells (Figure 2, C and D). TRAF3 overexpression also significantly reduced the protein level of *Mmp13* and COX-2 under the stimulation of IL-17 (Figure 2, E, G, and H). To further confirm the role of TRAF3 in IL-17–mediated induction of downstream genes, *Traf3*-specific siRNA were generated. These data showed that *Traf3* expression was reduced efficiently at both the mRNA and protein levels in primary chondrocytes (Supplemental Figure S1, C and D). As expected, TRAF3 loss of function enhanced IL-17–induced up-regulation of MMP-13 and COX-2 at the protein level (Figure 2, F, I, and J). Considering that TRAF3 interfered with the binding of IL-17 receptor with Act1, and consequently blocked the recruitment of TRAF6, finally resulting in a suppression of IL-17–mediated signaling and downstream gene transcription,²² three *Act1* specific siRNAs were therefore generated to knock down *Act1* in chondrocytes. siRNA3 was used for the following investigations because of its highest knock-down efficiency (Supplemental Figure S1, E and F). Importantly, knockdown of *Act1* dramatically reduced the up-regulation of the MMP-13 protein level induced by IL-17 (Supplemental Figure S1G). Collectively, these data solidly show that TRAF3 critically controls IL-17–induced downstream catabolic gene expression in chondrocytes.

IL-17 Deficiency Protects Cartilage from Degradation in Experimental and Spontaneous OA

Next, this study examined further whether blocking IL-17 signaling can protect cartilage from degradation *in vivo*. Interestingly, cartilage destruction triggered by DMM surgery was alleviated significantly in IL-17–deficient mice compared with littermate controls (Figure 3A). Furthermore, IL-17 depletion also notably mitigated cartilage degradation caused by age-related spontaneous OA (Figure 3B). Collectively, these results suggest that IL-17 plays a critical role in cartilage degradation during OA.

TRAF3 Transgenic Mice Show Less Matrix Loss Induced by IL-17

To investigate the role of TRAF3 in IL-17–induced cartilage degradation *in vivo*, T3TG mice and littermate controls were used to generate an IL-17 intra-articular injection model. MMP-13 is a critical catabolic factor during cartilage extracellular matrix loss and cartilage degradation. Indeed, an obvious increase of IL-17 injection-induced *Mmp13* production was detected in the superficial cartilage zone of WT mice, which was inhibited significantly in the cartilage of T3TG mice (Figure 4, A and B). Consistently, cartilage extracellular matrix loss, which was reflected by

safranin-O staining, caused by IL-17 injection, was attenuated in T3TG mice compared with littermate controls (Figure 4C). Taken together, these results proved that TRAF3 attenuates IL-17–induced MMP-13 production, and thus inhibits cartilage degradation *in vivo*.

TRAF3 Expression Is Reduced in OA Cartilage

To determine whether the TRAF3 expression level in articular cartilage is altered during OA, an experimental OA rat model was generated to investigate the expression of Traf3 in OA and normal cartilage. The immunohistochemistry results showed that the Traf3 expression level in experimental OA cartilage was slightly reduced 1 month after surgery and decreased significantly after 2 and 3 months compared with the sham group (Figure 5A). Meanwhile, the mRNA level of *Traf3* in cartilage was analyzed at different stages of the rat experimental OA model and showed that TRAF3 was reduced significantly during the OA process (Figure 5B). A similar observation that the TRAF3 expression level in OA cartilage was lower than that of normal cartilage also was found in human OA patients (Figure 5C). In summary, these observations indicate that TRAF3 is down-regulated in cartilage during OA pathogenesis.

TRAF3 Plays a Protective Role in Cartilage Degradation Caused by OA

Considering that TRAF3 has an inhibitory role in IL-17–mediated signaling and induction of matrix-degrading enzymes *in vitro*, this study further investigated whether TRAF3 affects experimental OA pathogenesis *in vivo*. To this end, an experimental OA model was generated by DMM surgery in T3TG mice and littermate controls. One month after the surgery, TRAF3 deficiency effectively alleviated cartilage destruction triggered by DMM surgery, which was shown by histology and Osteoarthritis Research Society International scoring (Figure 6A). To confirm further if TRAF3-mediated suppression of IL-17 signaling is responsible for its protective effect on cartilage degradation, Ad-sh-*Traf3* were injected intra-articularly into the knee joint of mice with DMM-induced OA. The results showed that loss of TRAF3 worsened cartilage destruction caused by surgery-induced OA, however, Ad-sh-*Traf3* had no effect on cartilage degradation caused by DMM surgery in *Il17a*^{−/−} mice (Figure 6B). In summary, our results suggest that TRAF3-mediated suppression of IL-17 signaling is a potential effective therapeutic target for cartilage matrix degradation in OA.

Discussion

IL-17 has been found to activate various signaling pathways for robust induction of proinflammatory cytokines and chemokines.^{4,13} Moreover, IL-17 also can act synergistically with IL-1 β or TNF- α for further induction of

proinflammatory cytokines, nitric oxide, and MMPs,¹⁴ as well as reduction of the proteoglycan level.²⁸ Indeed, IL-17, acting as an upstream factor, significantly increased the expression of IL-6, MMP-13, ADAMTS-4, and ADAMTS-5 in primary chondrocytes, the ATDC5 cell line, and the SW1353 cell line. However, the up-regulation of these catabolic factors induced by IL-17 is relatively modest compared with IL-1 β or TNF- α , suggesting that IL-17 might not be a direct regulator of these catabolic factors, rather, IL-17 might up-regulate IL-1 β or TNF- α through the activation of NF- κ B and MAPK pathways to turn on the expression of catabolic factors. Similarly, *in vivo* local administration of IL-17 induced MMP-13 production in articular cartilage, accompanied by cartilage matrix loss. However, only mild matrix loss was observed in the IL-17 injection model, which might be attributed to the short IL-17 exposure time course (3 days). Most importantly, IL-17 global loss-of-function mice showed attenuated cartilage degradation during surgery-induced OA and aging-related spontaneous OA, suggesting that IL-17 might be an important mediator of cartilage degradation during OA. Therefore, blocking IL-17–mediated downstream signaling in chondrocytes is a potent strategy to protect cartilage from degradation.

TRAF3 was identified as an important negative regulator of the noncanonical NF- κ B pathway in multiple cell types.^{19,29} A previous study showed that IL-17 stimulation induces the recruitment of TRAF3 to IL-17 receptor, which interferes with the formation of the IL-17 receptor–Act1–TRAF6 activation complex.²² A previous study showed that TRAF3 suppressed IL-17–mediated signaling in primary synoviocytes.²² Consistently, TRAF3 overexpression notably suppressed IL-17–mediated phosphorylation of p65, JNK, ERK, and p38, and consequent production of IL-6, MMP-13, Adamts-4, and COX-2, but not Adamts-5 in primary chondrocytes in the current study. However, TRAF3 overexpression only suppressed p65 and JNK phosphorylation in the ATDC5 cell line, indicating the heterogeneity of TRAF3 function in different cell types. To confirm the protective role of TRAF3 in IL-17–mediated cartilage degradation *in vivo*, an IL-17 intra-articular injection model was established. Importantly, TRAF3 gain-of-function effectively reduced IL-17–induced MMP-13 production and matrix loss in mice. Interestingly, TRAF3 overexpression does not affect the Adamts-5 expression level in both primary chondrocytes and the ATDC5 cell line. Adamts-5 was thought to be the main aggrecanase in mice,³⁰ therefore, TRAF3 gain of function might not be able to attenuate aggrecan degradation in mice.

The cartilage matrix loss and cartilage degradation of T3TG mice were attenuated compared with the WT mice. According to the Osteoarthritis Research Society International scoring, in T3TG mice, modest cartilage extracellular matrix loss still was observed after the surgery (grades 1 to 2). However, obvious cartilage degradation was not observed in T3TG mice, whereas the WT cartilage underwent notable degradation (grades 2 to 5).

In addition, the involvement of surface area in T3TG mice (stages 1 to 2) was less than that in the WT mice (stages 3 to 4). On the other hand, TRAF3 loss of function enhanced cartilage destruction caused by DMM surgery. Interestingly, the TRAF3 expression level decreased significantly at the late stage (from 2 months after DMM surgery) of OA pathogenesis but not the early stage in rat OA cartilage. By 3 months after DMM surgery, TRAF3 barely was detected in articular cartilage (Figure 5A). The mRNA expression further confirmed the significant change of TRAF3 during OA, suggesting that TRAF3 is down-regulated transcriptionally and leads to enhanced IL-17 signaling in cartilage during OA pathogenesis. These findings indicate that TRAF3 is a critical negative regulator of cartilage degradation during OA. More importantly, genetic deletion of IL-17 blocked TRAF3 knockdown-mediated promotion of cartilage destruction. These data suggest that the protective effect of TRAF3 on cartilage is mediated by its suppression of IL-17-mediated signaling.

In summary, we show that TRAF3, as a negative regulator of IL-17-mediated signaling, plays an important role in protecting articular cartilage from degradation during OA. The protective effect of TRAF3 on cartilage is partially owing to the suppression of IL-17-induced signaling and consequent production of cartilage catabolic factors. Thus, TRAF3 is a potential therapeutic target for the development of a treatment for cartilage degradation caused by OA.

Acknowledgment

We thank Prof. Youcun Qian (Chinese Academy of Sciences) for kindly providing the T3TG and *Il17a*^{-/-} mice.

Author Contributions

G.H. and N.Z. conceived and designed the study, wrote the manuscript, and collected, analyzed, and interpreted the data; J.L. collected and analyzed the data; J.W. performed animal surgery and collected data; W.W. collected human cartilage samples; J.L. wrote the manuscript; W.T. and X.Z. collected and analyzed the data; L.D. analyzed the data; and X.Z. conceived and designed the study, wrote the manuscript, and analyzed and interpreted the data.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.04.016>.

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