Blocking TNFα attenuates progressive cartilage matrix degradation in inflammatory arthritis

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Abstract. Because damage to hyaline cartilage is irreversible, relieving progressive cartilage destruction is an important therapeutic approach for inflammatory arthritis. In the present study, human hyaline chondrocytes were isolated from total knee replacements of 15 patients with osteoarthritis (OA) and three with rheumatoid arthritis (RA). Synovial fluid of OA (n=25) and RA (n=34) were collected to measure tumor necrosis factor α (TNFα) using ELISA. Consistent with previous studies, the synovial fluid exhibited high TNFα levels and hyaline cartilage was severely destroyed in patients with RA. TNFα-treated chondrocytes were used as model for inflammatory arthritis. TNFα did not influence proliferation or extracellular matrix expression in chondrocytes, but induced matrix metalloproteinase (MMP)1, 3 and 13 expression levels in chondrocytes, which was accompanied by activation of nuclear factor-κB signaling. During chondrogenic differentiation, TNFα attenuated mRNA expression levels of anabolic factors (collagen type 2 and aggrecan) and enhanced mRNA expression of catabolic factors (*MMP1*, *MMP3* and *MMP13*) in chondrocytes. Moreover, anti-TNFα agents (Golimumab) inhibited the TNFα-induced metabolic shift in chondrocytes and chondrogenic differentiation. The present study revealed a mechanism by which TNFα may induce metabolic shift in chondrocytes, leading to progressive chondrocyte destruction.

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Introduction

Hyaline cartilage can be damaged by trauma and is degraded in different forms of arthritis (1,2). Once damaged, cartilage has a limited capacity to repair and does not fully regenerate (3,4). In past decades, efforts have been made to achieve functional repair of hyaline cartilage and to regenerate hyaline cartilage-like tissue, but these clinical attempts have consistently failed (5,6). This may be due to a lack of knowledge regarding the formation and maintenance of distinct features of hyaline cartilage and the components of its extracellular matrix. Therefore, a biological understanding of chondrocytes derived from human joints and extensive research may be necessary to achieve these goals.

Cartilage is an unusual tissue in that chondrocytes can be anabolic (synthesize matrix) or catabolic (degrade matrix) (7). In adult cartilage, a balance exists between the synthesis and degradation of the cartilaginous extracellular matrix. However, inflammation can cause an imbalance between expression of anabolic [aggrecan (ACAN) and collagen type II (COL2)] and catabolic [matrix metalloproteinases (MMPs)] factors in chondrocyte tissue (8-10). A severe imbalance between anabolic and catabolic chondrocyte factors leads to progressive degradation of the cartilage matrix (11-13). Specifically, pro-inflammatory stimuli in chondrocytes drive poor regenerative capacity by inducing catabolic molecules (14).

Cartilage destruction is a key characteristic of degenerative joint diseases, particularly osteoarthritis (OA); it also features in chronic inflammatory joint diseases, such as rheumatoid

arthritis (RA) (2). Tumor necrosis factor α (TNFα) is a pleotropic cytokine that plays key regulatory roles in inflammation and the response to cellular stressors such as oxidative stress (15,16). It also serves a vital role in the pathological process of inflammatory changes by promoting release of MMP and downregulation of SRY-box transcription factor 9 (SOX9), COL2 and ACAN expression, eventually leading to extracellular matrix destruction in hyaline cartilage (17). Therefore, TNFα has been highlighted as a pathological factor in both OA and RA. Previous studies have reported that TNFα serves an important role in hyaline cartilage destruction in both OA and RA pathophysiology (18,19). Therefore, TNFα has proven to be a potential effective therapeutic target for patients with RA (15).

The present study defined specific surface markers of primary chondrocytes derived from human OA hyaline cartilage and investigated the role of TNFα in the chondrogenesis of hyaline cartilage.

Materials and methods

Human OA and RA subjects. The present study was performed in accordance with Hanyang University Hospital Institutional Review Board guidelines and approved by the Ethics Committee of Hanyang University Hospital (approval no. 2017-05-003) and Hanyang University Guri Hospital (approval no. 2018-07-024). Written informed consent was obtained from all subjects.

Between April 2018 and May 2019, 15 patients with OA (9 females and 6 males; mean age, 70.5±8.7 years) and three with RA (all females; mean age, 57.1±11.3 years) were enrolled and surgical samples were obtained from total knee replacement at Hanyang University Guri Hospital. Both OA and RA surgical knee samples were fixed at room temperature (RT) for two weeks with 10% formalin, decalcified with 10% formic acid and embedded routinely in a paraffin block. The paraffin block was sectioned at a thickness of 3.5 µm and stained by hematoxylin and eosin, Safranin O and Toluidine blue. The section slides were deparaffinized in the tissue in 100% neoclear (cat. no. 109843; Merck) for 10 min and rehydration in serial ethanol dilution (100, 90, 80, 70, and 50% ethanol) for 2 min each step, and washed with tap water for 5 min. For H&E staining, the slides were stained with 100% hematoxylin for 3 min, washed with tap water for 10 min, 100% eosin stained for 3 min, washed with tap water, and mounted with a permanent mounting medium (cat. no. H-5000; Vector Lab). For Safranin O staining, the slides were stained with 0.1% fast green stained for 5 min, washed with 1% acetic acid one tapping, washed with tap water for 1 min, 0.1% Safranin O for 6 min, washed with tap water for 1 min, and mounted with a permanent mounting medium. For toluidine blue staining, the slides were stained with 0.1% toluidine blue stained for 2 min, washed with tap water for 10 min, and mounted with a permanent mounting medium. All staining procedures performed at RT. Stained slides were imaged by a Nikon eclipse Ti-U light microscope (Nikon Corporation).

Between February 2017 and May 2019, synovial fluid samples were collected from 34 patients with OA (eight men and 26 women; mean age, 53.7±16.1 years) and 25 with RA (all women; mean age, 68.6±8.5 years) at Hanyang University Hospital for Rheumatic Disease. For synovial fluid analysis, 10 ml fluid was incubated with 1.5 mg hyaluronidase (cat. no. H3506; Sigma-Aldrich) for 15 min at 37˚C, followed by centrifugation at 1,400 x g for 15 min at 4˚C. After centrifugation, the fluids were immediately divided into aliquots and stored at -80˚C for TNFα levels (cat. no. DTA00D; R&D Systems, Inc.) measurement using ELISA according to manufacturer's protocol.

Isolation of human OA chondrocytes. Hyaline cartilage from OA knee joints was scraped using a rongeur and collected in serum-free DMEM (cat. no. L0103-500; Biowest) buffer containing 1 mg/ml Collagenase type 2 (cat. no. C6885; Sigma-Aldrich). The collected tissue samples were incubated at 37˚C with agitation overnight. The next day, the digested hyaline cartilage was filtered through a 70-µm strainer (cat. no. 93070; SPL Life Sciences) and seeded ~60% cell density in DMEM (cat. no. L0103-500; Biowest) supplemented with 10% FBS (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin and streptomycin (cat. no. 15140122; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C and 5% CO_2. Primary chondrocytes at passage 2-5 were used in subsequent experiments. Until the passage 2-5, chondrocytes were cultured at 60% density before full cell density at 37˚C.

Reagents and biological agents. Recombinant human TNFα (cat. no. 300-01A; PeproTech, Inc.), golimumab (Janssen Global Services, LLC; 1 µg/ml, 37˚C, 4 weeks) and BAY 11-7082 (cat. no. B5556; Sigma-Aldrich; 10 or 30 µM, 37˚C, 24 h) were obtained.

Flow cytometric analysis. The chondrocytes were fixed using a fixation/permeabilization solution kit (cat. no. 554715; BD Biosciences) and stained with CD34 (cat. no. 343607; BioLegend, Inc.; 1:100), CD44 (cat. no. 338807; BioLegend, Inc.; 1:100), CD59 (cat. no. 304711; BioLegend, Inc.; 1:100), CD74 (cat. no. 326811; BioLegend, Inc.; 1:100), CD90 (cat. no. 328109; BioLegend, Inc.; 1:100), CD105 (cat. no. 323205; BioLegend, Inc.; 1:100), CD146 (cat. no. 361015; BioLegend, Inc.; 1:100), CD164 (cat. no. 324805; BioLegend, Inc.; 1:100), SOX9-Alexa-647 (cat. no. 565493; BD Pharmingen; BD Biosciences; 1:200), ACAN-PE (cat. no. sc-33695; Santa Cruz Biotechnology, Inc.; 1:100), IgG1-Alexa-647 (cat. no. 557732; BD Pharmingen; BD Biosciences; 1:100), IgG1-PE (cat. no. 400112; BioLegend, Inc.; 1:100), IgG1-APC (cat. no. 400122; BioLegend, Inc.; 1:100), IgG1-FITC (cat. no. 400109; BioLegend, Inc.; 1:100) or IgG2a-APC (cat. no. 400221; BioLegend, Inc.; 1:100) for 30 min at 4˚C. The dilution of the antibody for FACS was ranged from 1:100-1:200. After staining, cells were washed with Perm/Wash Buffer (cat. no. 554715; BD Biosciences) and analyzed by flow cytometry (FACS Canto II; BD Biosciences). Data were analyzed using FlowJo version 10.7 software (FlowJo LLC).

Proliferation assay. The water-soluble tetrazolium salt (WST) assay was performed with EZ-CYTOX (cat. no. EZ-1000; Dogen Bio Co., Ltd.) according to the manufacturer's instructions. Primary chondrocytes were plated into 96-well plate ($1x10^3$ cells/well) and treated with 10 or 25 ng/ml TNFα

in 37˚C CO_2 incubator for 1-6 days. WST solution was added to the cells, which were incubated for 1 h. The absorbance at 450 nm was measured with a microplate reader (Thermo Fisher Scientific, Inc.).

Human MMP antibody array. Chondrocytes were stimulated with 10 ng/ml human TNFα at RT for 1 day and collected for human MMP analysis. The stimulated cells were lysed in 1X RIPA buffer including phosphatase and proteinase inhibitors and assessed according to the manufacturer's protocol (cat. no. ab134004; Abcam). To analyze the array data, comparison of signaling intensities for individual spots was detected using the UVItech system (Cleaver Scientific Ltd.) and analyzed with ImageJ 1.52a version software (National Institutes of Health).

Reverse transcription-quantitative (RT-q)PCR. RT-qPCR was performed as previously described (20). Briefly, total RNA of the TNF-treated cells extracted with TRIzol® reagent (cat. no. 15596018; Invitrogen; Thermo Fisher Scientific, Inc.) was used to generate complementary DNA (42˚C for 1 h and then 70˚C for 10 min) using a RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.). qPCR was performed on a CFX96 Real-time PCR detection system (cat. no. 1855201; Bio-Rad Laboratories, Inc.) following the manufacturer's procedure and the following thermal cycling: Initial denaturation for 3 min at 95˚C; two step-cycling: Denaturation for 10 sec at 95˚C, combined annealing/extension for 30 sec at 60˚C for 35 cycle. The expression of each target gene was normalized to *GAPDH*. Normalized expression values were averaged and the relative levels of gene expression were quantified by using the comparative CT method (21). Primers used for PCR were as follows: *MMP1* forward, 5'-AGAGCAGATGTGGACCATGC-3' and reverse, 5'-TTGTCCCGATGATCTCCCCT-3'; *MMP3* forward, 5'-TCTATGGACCTCCCCCTGAC-3' and reverse, 5'-GATTTGCGCCAAAAGTGCCT-3'; *MMP13* forward, 5'-GCCATTACCAGTCTCCGAGG-3' and reverse, 5'-TAC GGTTGGGAAGTTCTGGC-3'; *SOX9* forward, 5'-CTGAAC GAGAGCGAGAAGCG-3' and reverse, 5'-CCCGTTCTTCAC CGACTTCC-3'; *ACAN* forward, 5'-TGGGAACCAGCCTAT ACCCCAG-3' and reverse, 5'-CAGTTGCAGAAGGGCCTT CTGTAC-3'; *COL2* forward, 5'-GCCGGATCTGTGTCTGTG AC-3' and reverse, 5'-TGTCCCTTTGGTCCTGGTTG-3'; and *GAPDH* forward, 5'-CAAGATCATCAGCAATGCC-3' and reverse, 5'-CTGTGGTCATGAGTCCTTCC-3'.

Immunoblotting. Immunoblot analysis was performed as previously described (22). The treated cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.6% Na-deoxycholate, 1% Triton X-100) supplemented with protease (cat. no. 535140, Calbiochem) and phosphatase (cat. no. 5870, Cell signaling) inhibitor cocktails. Lysed samples were incubated on ice for 1 h followed by a centrifugation at 12,000 x g for 30 min at 4˚C. The protein of whole lysates were determined using a Bradford protein assay (cat. no. 5000006; Bio-Rad Laboratories, Inc.). Protein (30~50 µg) were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (cat. no. 10600002, Cytiva) in a transbuffer. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% Tween-20 and incubated with specific primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The dilution of the primary antibody for Immunoblotting was ranged from 1:500 to 1:1,000. Diluted primary antibodies were incubated at 4˚C overnight and secondary antibodies incubation diluted 1:1,000 at RT for 1 h. Membranes were visualized with Pierce ECL (cat. no. 34580; Thermo Fisher Scientific, Inc.) and the visualized images were collected by chemiluminescence imaging system (Alliance Q9 advanced, Uvitech System). The primary antibodies for TNF receptor 1 (cat. no. sc-8436; 1:1,000) and NF-κB p65 (cat. no. sc-372; 1:1,000) were from Santa Cruz Biotechnology, Inc. Phosphorylated (p-)NF-κB p65 (cat. no. 3033; 1:1,000), p-ERK (cat. no. 9101s; 1:1,000), total-ERK (cat. no. 9102s; 1:1,000), p-p38 (cat. no. 9215s; 1:500), total-p38 (cat. no. 9212; 1:1,000), and β-actin (cat. no. 4970; 1:5,000) antibodies were from Cell Signaling Technology, Inc. MMP-1 (cat. no. MAB901; 1:500), MMP-3 (cat. no. MAB513; 1:500), and MMP-13 (cat. no. MAB511; 1:500) antibodies were from R&D Systems, Inc. Goat anti-rabbit (cat. no. 111-035-003; 1:2,000) and anti-mouse (cat. no. 115-035-003; 1:2,000) secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc.

Trichloroacetic acid (TCA) assay. TCA precipitation was performed as previously described (23). In brief, chondrocytes were seeded at 80-90% confluence. The next day, growth medium was replaced with serum-free DMEM including 10 ng/ml TNFα or distilled water for 1 day. The cell supernatant obtained by centrifugation at 1,224 x g at 4˚C for 10 min was collected for TCA precipitation, subjected to 12.5% SDS-PAGE and then stained with Coomassie Brilliant Blue R-250 solution (cat. no. C2006; Biosesang) as a loading control. After destaining (acetic acid: Methanol: Water; 7.5:5:87.5) overnight, the gel was washed five times with distilled water for 10 min and followed by immunoblotting procedures.

Immunofluorescence. The TNFα-stimulated chondrocytes were washed twice with 1X PBS and fixed with 10% formalin at RT for 15 min, followed by permeabilization with 1X PBS containing 0.1% Triton X-100 and 1% BSA (cat. no. BSA-BSH-1XG; Rocky Mountain Biologicals, Inc.) at RT for 1 h, incubation with a primary antibody at 4˚C overnight, washing with 1X PBS and incubation with Cy3-conjugated anti-rabbit antibody (cat. no. 111-165-144; Jackson Immunoresearch) or Alexa 488-conjugated anti-mouse antibody (cat. no. A-11001; Invitrogen) for 1 h. All primary and secondary antibodies were used at 1:100 dilution. The stained cells were washed with distilled water and mounted with DAPI (cat. no. H1200; Vector Laboratories, Inc.; Maravai Life Sciences). In order to visualize stained cells, immunofluorescence images were collected with a confocal microscope (TCS SP5; Leica Microsystems GmbH). Images were captured using LAS version 4.2.1 software (Leica Microsystems GmbH).

Promoter assay. NF-κB p65 wild-type and two p65 S536A (substitution of alanine for serine) or S536E (substitution of glutamate for serine 536) mutant promoters in pGL3-Basic were a gift from Dr Heekyoung Chung (Hanyang University,
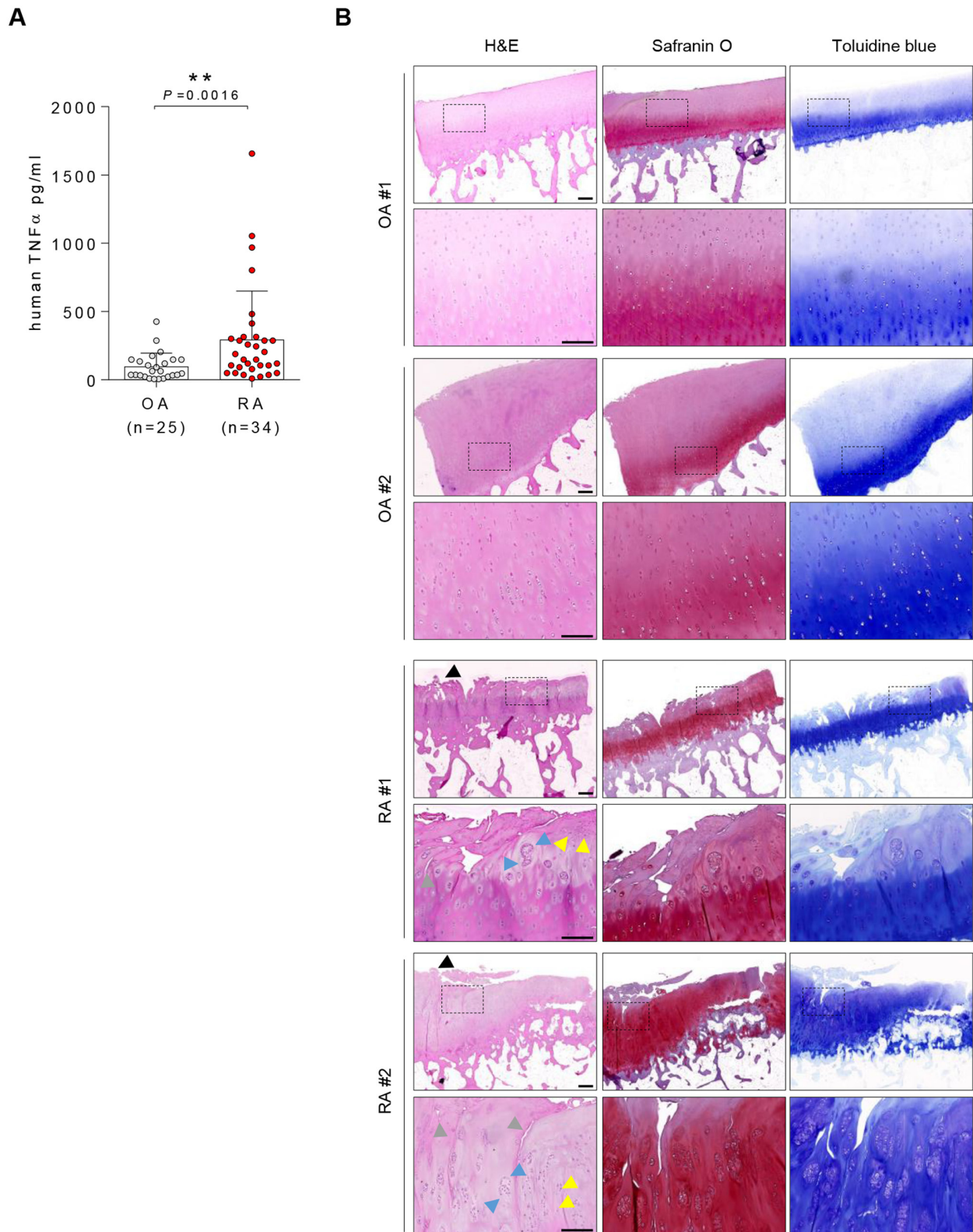
Figure 1. High TNFα levels in synovial fluid and destruction of hyaline cartilage are observed in RA. (A) TNFα levels in OA (n=25) and RA (n=34) synovial fluid were assessed by ELISA. [**]P<0.01, unpaired Student's t-test. (B) Knee tissue samples (including hyaline cartilage) from patients with OA and RA were stained with H&E, Safranin O and Toluidine Blue. Overt fibrillation indicated by black arrows; Chondyocyte clustering indicated by blue arrows; changes in chondrocyte morphology/distribution indicated by yellow arrows; matrix destruction indicated by grey arrows. Scale bar, 200 µm. RA, rheumatoid arthritis; OA, osteoarthritis; H&E, hematoxylin and eosin; TNFα, tumor necrosis factor α.
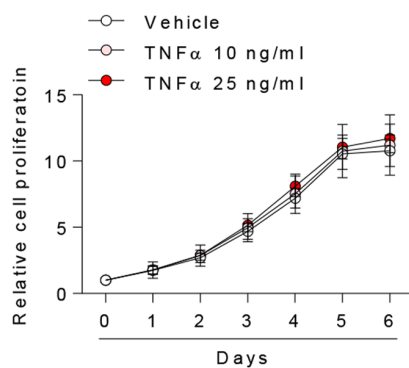
Seoul, South Korea) (24). 293T cells were a generous gift from Dr Heekyoung Chung (Hanyang University, Seoul, Republic of Korea) and seeded into 60-mm culture plates (2x10^5 cells per well) and co-transfected with p65 wild-type, p65 S536A or p65 S536E (1 µg/well), and *Renilla* (0.25 µg/well) plasmids as control for 48 h using Lipofectamine® 3000
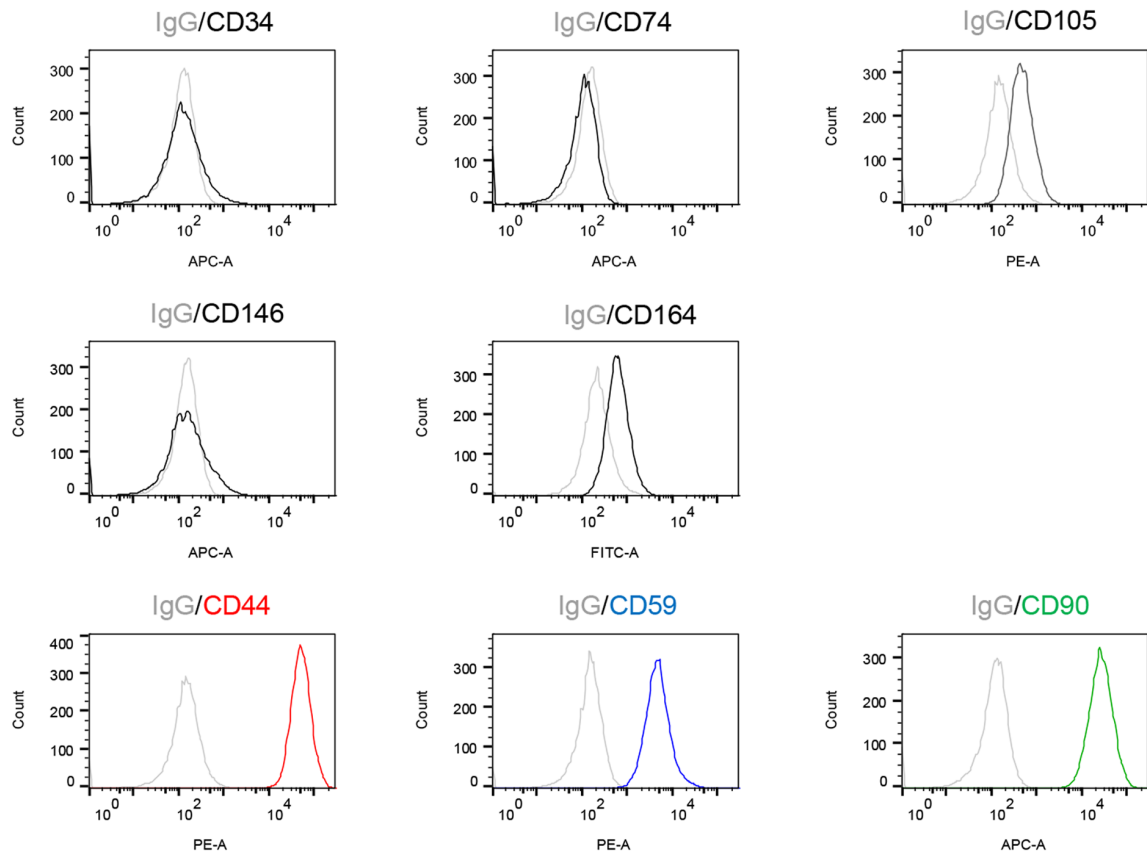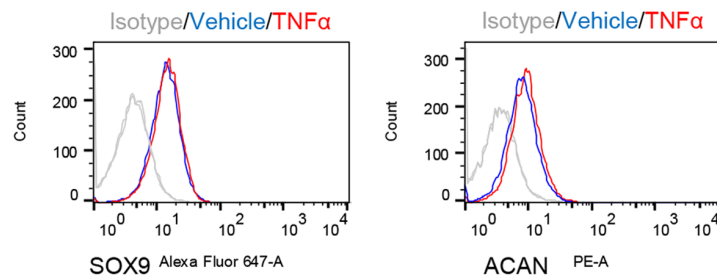
Figure 2. TNFα does not significantly affect proliferation and extracellular molecule expression in OA chondrocytes. (A) CD34, CD44, CD59, CD74, CD90, CD105, CD146 and CD164 surface markers of chondrocytes were evaluated by flow cytometry. (B) Chondrocytes were exposed to TNFα and assessed by water-soluble tetrazolium salt assay. n=5, one-way ANOVA with Tukey's post hoc test. (C) Chondrocytes were exposed to 25 ng/ml TNFα for 24 h and assessed by flow cytometry. IgG was used as a control. OA, osteoarthritis; TNFα, tumor necrosis factor α; ACAN, aggrecan; SOX9, SRY-box transcription factor 9.

(cat. no. L3000-015; Invitrogen; Thermo Fisher Scientific, Inc.). The transfected cells were reseeded on a 12-well culture plate (5×10^4 cells per well) for treatment at 37°C for 24 h with distilled water or 10 or 25 ng/ml TNFα and then analyzed with Dual-Luciferase Reporter Assay system (cat. no. E1500; Promega Corporation). The procedure was performed according to the manufacturer's instructions and activity was measured with a luminometer (Titertek-Berthold). The measured values were analyzed by comparison with *Renilla* luciferase. This ratio was then normalized to the averaged ratio of vehicle.

Chondrogenic differentiation of chondrocytes with pellet culture. The 'pellet culture' method was performed as previously described (25). Briefly, primary chondrocytes (2×10^5) were collected following centrifugation at 25°C for 3 min at 441 x g, replaced and cultured in chondrogenic medium with distilled water or TNFα for 4 weeks. The medium was changed every 3 days. Chondrogenic medium was composed of serum-free DMEM/F12 (cat. no. 11320033; Gibco; Thermo Fisher Scientific, Inc.) with 10% Insulin-Transferrin-Selenium premix tissue culture supplement (cat. no. I3146; Sigma-Aldrich; Merck KGaA), 10 μM dexamethasone (cat. no. D-2915;
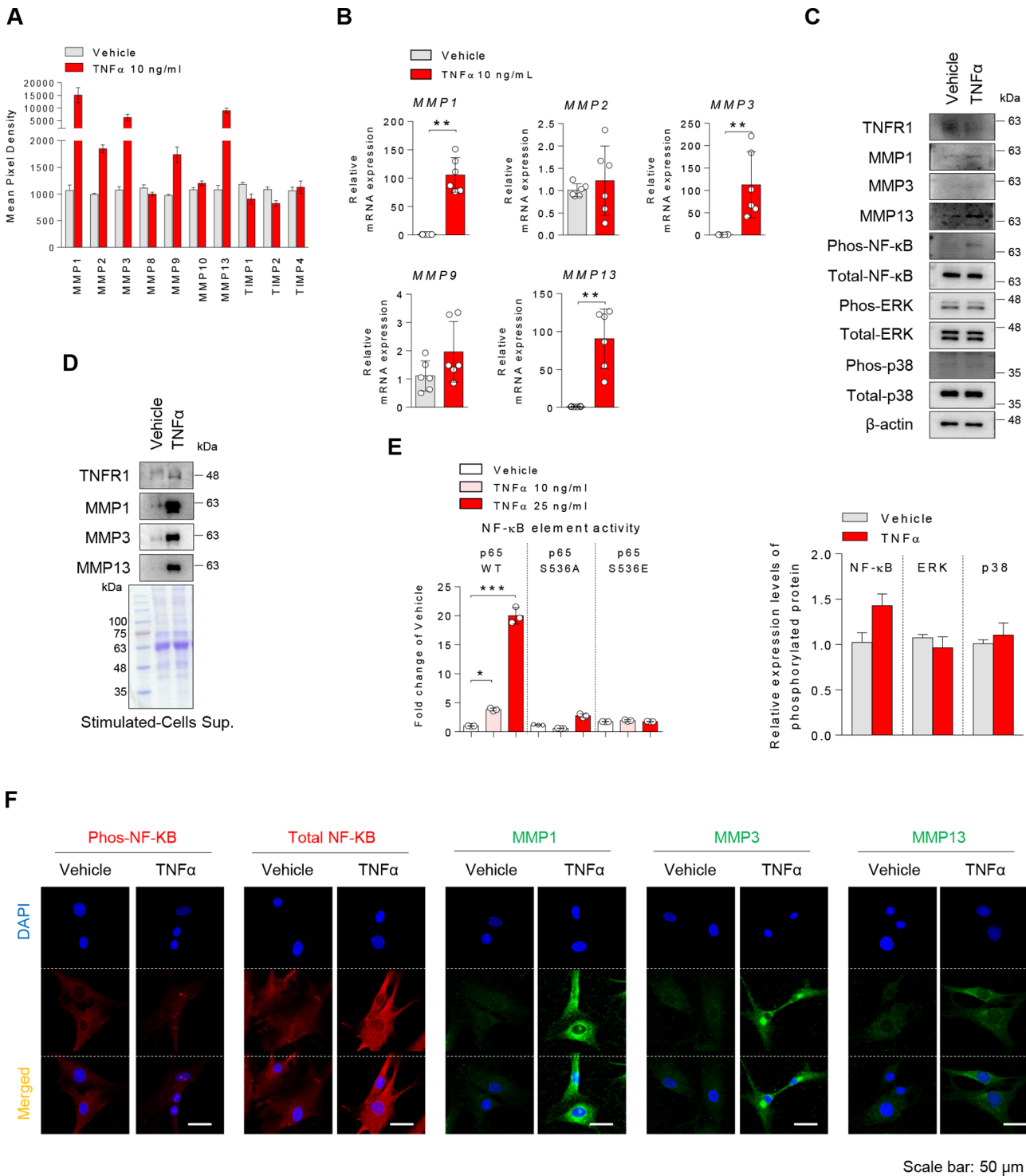
Figure 3. TNFα induces the expression of MMPs in OA chondrocytes via NF-κB activation. (A) Chondrocytes were treated with vehicle or 10 ng/ml TNFα for 24 h. MMP protein expression levels in stimulated cell lysates were semi-quantified via a human MMP antibody array (n=2). (B) *MMP* mRNA expression in stimulated cells was determined by reverse transcription-quantitative PCR. (C) TNFα-treated chondrocytes were assessed by immunoblotting. Phosphorylation was semi-quantified with ImageJ and calculated relative to each total protein. (D) Proteins secreted in the cell supernatant by TNFα stimulation were precipitated with trichloroacetic acid and detected by immunoblotting. Coomassie blue staining was used as a loading control. (E) 293T cells were transfected with p65 wild type or double mutants (S536S or S536E), followed by treatment with vehicle, 10 or 25 ng/ml TNFα for 24 h; promoter activity analysis was subsequently performed. n=3, one-way ANOVA with Tukey's post hoc test. (F) Protein expression levels of p-NF-κB, total NF-κB and MMP1, 3 and 13 in TNFα-stimulated cells were analyzed by immunofluorescence. Representative data are shown. Scale bar, 50 μm. *P<0.05, **P<0.01; ***P<0.001 (mean ± SD; n=6). OA, osteoarthritis; phos, phosphorylated; TNFα, tumor necrosis factor α; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB.

Sigma-Aldrich; Merck KGaA), 1 μM ascorbate-2-phosphate (cat. no. 49752; Sigma-Aldrich; Merck KGaA), 1% sodium pyruvate (cat. no. 11360070; Gibco; Thermo Fisher Scientific, Inc.) and 10 ng/ml TGF-β1 (cat. no. 0218209-1; PeproTech, Inc.). 10% Formalin-fixed pellets at RT for 24 h were washed with 1X PBS and transferred to 30% sucrose solution overnight.
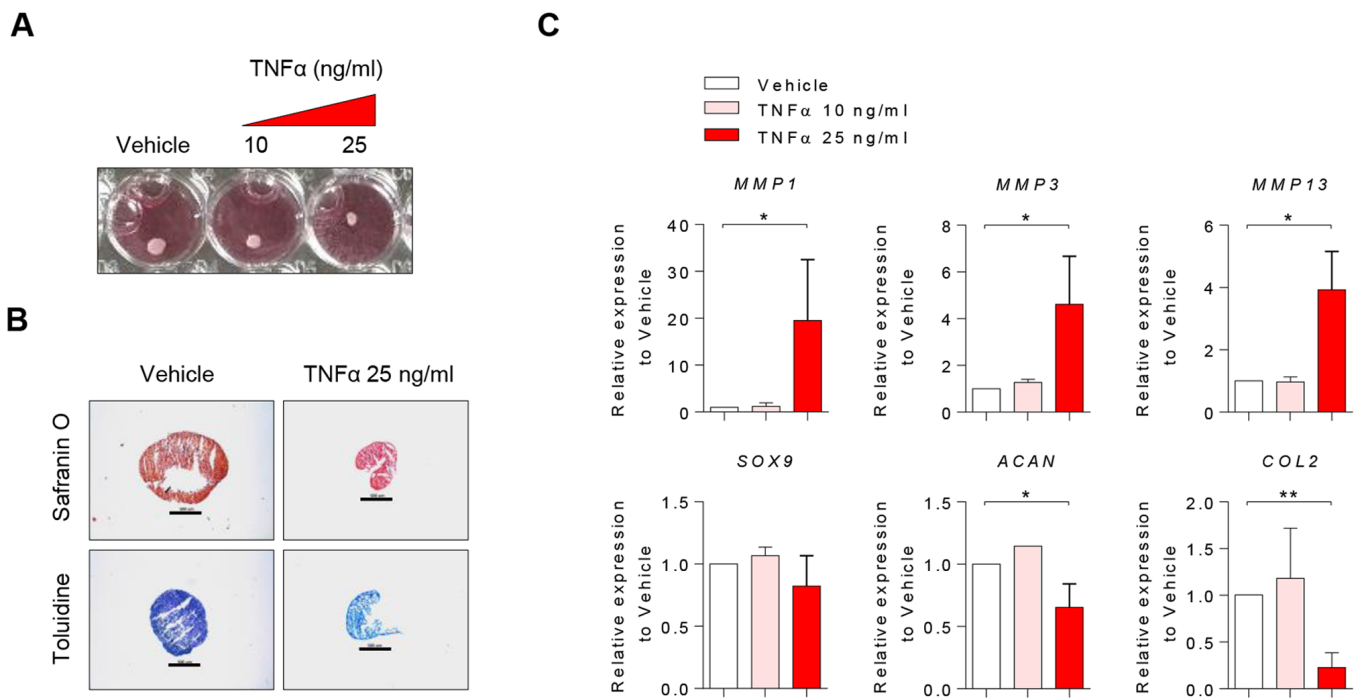
Figure 4. TNFα inhibits chondrogenic differentiation of osteoarthritis chondrocytes. Chondrocytes were induced with chondrogenic differentiation media for 28 days in the presence of vehicle, or 10 or 25 ng/ml TNFα. (A) Images were captured after 28 days differentiation. Differentiating chondrocytes were (B) stained by Safranin O and Toluidine Blue and (C) analyzed by reverse transcription-quantitative PCR. Scale bar, 500 μm. Data are presented as the mean ± SD (n=4). *P<0.05; **P<0.01. COL2, collagen type 2; ACAN, aggrecan; MMP, matrix metalloproteinase; SOX9, SRY-box transcription factor 9; TNFα, tumor necrosis factor α.

Pellets were soaked in OCT compound (cat. no. 4583; Sakura Finetek USA) and sectioned on a cryotome (cat. no. CM1850; Leica Microsystems GmbH) to create 15-μm sections on gelatin-coated slides. Then, the slides were stained with Safranin O (cat. no. 1446640250; ACROS Organics) and Toluidine blue (cat. no. T3260-5g; Sigma-Aldrich; Merck KGaA). Each staining was washed with water for 1 min and then stained with 0.1% dyeing solution for 1 min at RT. Stained slides were imaged under a Nikon eclipse Ti-U light microscope (Nikon Corporation).

Statistical analysis. Data were analyzed with GraphPad Prism 6 software (GraphPad Software, Inc.). A two-tailed Student t-test was used to compare data between two unpaired groups. One-way ANOVA with Tukey's post hoc test was used to compare data between more than two groups. All data are expressed as the mean ± SD (n≥3). P<0.05 was considered to indicate a statistically significant difference.

Results

High TNFα levels in synovial fluid and destruction of hyaline cartilage are observed in RA. TNFα levels in synovial fluid were significantly higher in patients with RA than OA (mean, 168.7 vs. 64.28 pg/ml; Fig. 1A). Although superficial fibrillation and loss of proteoglycan detection with safranin O staining were observed in OA hyaline cartilage, patients with RA exhibited more severe hyaline cartilage damage than patients with OA. Overt fibrillation (black arrows), chondrocyte clustering (blue arrows), changes in chondrocyte morphology/distribution (yellow arrows) and matrix destruction (grey arrows) were observed in patients with RA (Fig. 1B).

TNFα has no significant effects on proliferation and extracellular molecule expression in OA chondrocytes. The present study aimed to detect known chondrocyte surface markers and investigate the effects of TNFα on them (22,23). CD44, CD59 and CD90 were highly expressed in chondrocytes, whereas CD34, CD74 and CD146 were not; in addition, there was partial positive expression of CD105 and CD164. (Fig. 2A). Following TNFα treatment, there were no significant changes in the rate of cell proliferation (Fig. 2B) or chondrocyte expression of SOX9 and ACAN (Fig. 2C).

TNFα induces MMP expression in human OA chondrocytes. TNFα-treated chondrocytes were used as a model for inflammatory arthritis. In order to determine whether TNFα may affect the destructive hyaline cartilage of patients with inflammatory arthritis, chondrocytes were stimulated with TNFα. To confirm dose effects of TNFα in chondrocytes, we treated with 1, 5, 10, 25, 50 ng/ml TNFα dose and analyzed MMP expression levels using RT-qPCR and immunoblotting. We confirmed an increase in MMP1, 3, 13 expression in dose-dependently TNFα treatment (Fig. S1). Strong increases in the protein expression levels of MMP1, 3 and 13, and a decrease in TIMP metallopeptidase inhibitor (TIMP)1 and TIMP2 expression levels were observed (Figs. 3A, S2 and S3). Changes in the expression levels of MMPs were validated by RT-qPCR (Fig. 3B) and immunoblotting (Fig. 3C). TNFα stimulation of chondrocytes elevated MMP1, 3, and 13 protein expression levels in the cytoplasm and led to extracellular secretion of these proteins (Fig. 3C, D and F).
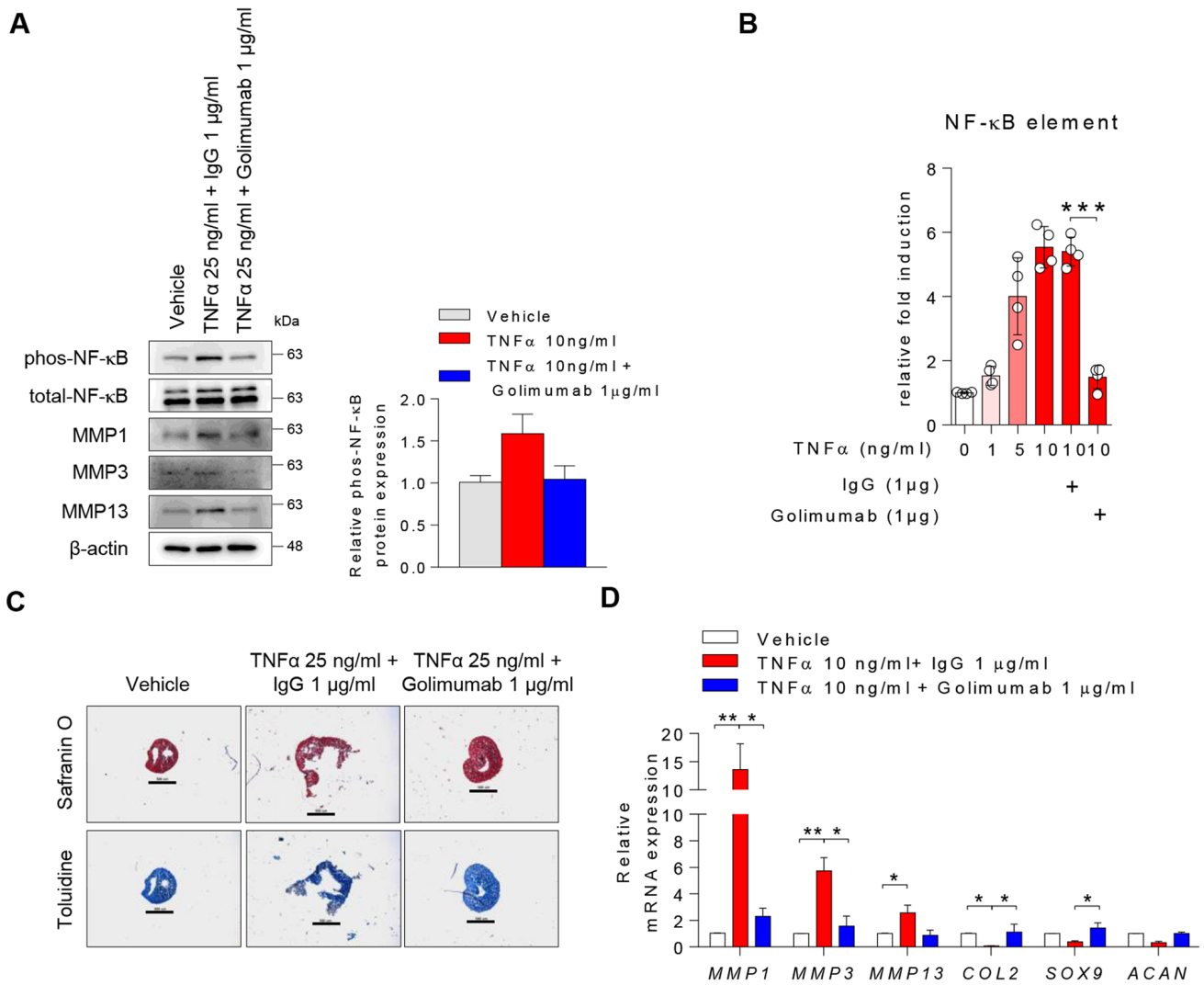
Figure 5. Blocking TNFα attenuates TNFα-driven degradation of the extracellular matrix of osteoarthritis chondrocytes. (A) Chondrocytes were treated for 24 h and analyzed by immunoblotting. Golimumab was used as an anti-TNF agent (n=3). P-NF-κB protein was semi-quantified with ImageJ and calculated relative to total NF-κB protein. (B) 293T cells were co-transfected with NF-κB promoters and *Renilla* plasmids, incubated for 48 h and then subjected to luciferase activity assay. (C) Chondrocytes were stimulated with TNFα or a TNFα blocker during chondrogenic differentiation for 28 days and then analyzed by Safranin O and Toluidine Blue staining. (D) mRNA expression was analyzed by reverse transcription-quantitative PCR. n=3, *P<0.05; **P<0.01; ***P<0.001, one-way ANOVA with Tukey's post hoc test. phos, phosphorylated; COL2, collagen type 2; ACAN, aggrecan; MMP, matrix metalloproteinase; SOX9, SRY-box transcription factor 9; TNFα, tumor necrosis factor α.

Moreover, TNFα induced NF-κB phosphorylation and nuclear translocation in chondrocytes, and significantly augmented p65 promoter activity, but not that of mutants (Fig. 3E and F). Therefore, it was concluded that TNFα promoted the expression of MMP1, 3 and 13 in human chondrocytes, which was accompanied by activation of NF-κB signaling.

TNFα decreases the regenerative capacity of OA chondrocytes during differentiation. In order to elucidate the effect of TNFα on regenerative capacity in chondrocytes, chondrogenic differentiation of chondrocytes was induced in the presence or absence of TNFα treatment. Chondrocytes treated with TNFα exhibited lower differentiation capacity; they showed weaker intensity of staining and smaller pellet size compared with controls (Fig. 4A and B). Furthermore, upregulation of *MMP1*, *MMP3* and *MMP13*, and downregulation of *ACAN* and *COL2* was observed (Fig. 4C), which suggested that TNFα stimulation induced a metabolic shift from anabolism

to catabolism during chondrogenic differentiation. Thus, it was concluded that TNFα decreased the regenerative capacity of chondrocytes during differentiation.

Blocking TNFα attenuates TNFα-driven cartilage matrix degradation in OA chondrocytes. To determine whether the modulation of TNFα affected catabolic and anabolic gene expression levels, human chondrocytes were treated with 1 μg/ml golimumab (a TNFα blocker) in the presence of TNFα. TNFα stimulation upregulated p-NF-κB, MMP1, MMP3 and MMP13 in chondrocytes, whereas treatment with golimumab impeded these changes mediated by TNFα (Fig. 5A). In the line with Fig. 5A, changes in TNF-mediated p-NF-κB were quantified (Fig. 5A, right panel). TNFα activated the WT NF-κB promoter in a dose-dependent manner but blocking TNFα diminished this activation (Fig. 5B). In addition, treatment with 10 or 30 μM BAY, an NF-κB inhibitor, suppressed TNFα-mediated induction of *MMP1*, *MMP3* and *MMP13* mRNA expression in

chondrocytes (Fig. S4). During chondrogenic differentiation, treatment with the TNFα blocker interfered with the destructive effect of TNFα on physical changes of chondrogenesis (Fig. 5C) and its mRNA expressions (Fig. 5D). Therefore, blocking TNFα may attenuate TNFα-driven cartilage matrix degradation in inflammatory arthritis.

Discussion

The present study revealed that TNFα levels were increased in synovial fluid and the destruction of hyaline cartilage was greater in RA compared with in OA. To demonstrate the association between TNFα and hyaline cartilage destruction in OA, chondrocytes were stimulated with TNFα; the results indicated that TNFα induced a metabolic shift in chondrocytes via NF-κB signaling. Based on these findings, a model for the pathogenesis of hyaline cartilage degradation in inflammatory arthritis was proposed. TNFα, an inflammatory cytokine that is increased by excessive inflammation, may modulate anabolic and catabolic factors, thereby revealing potential therapeutic targets for inflammatory arthritis progression.

The precise mechanism by which TNFα suppresses the expression of matrix proteoglycans, such as ACAN and COL2, requires further study. It was hypothesized that MMP 1, 3 and 13 are indicators of TNFα-driven matrix proteoglycan degradation. Although 24-h TNFα exposure did not affect SOX9 and ACAN expression in chondrocytes, exposure to TNF during chondrogenic differentiation resulted in decreased mRNA expression levels of anabolic mediators, such as *ACAN* and *COL2*. Exposure of chondrocytes to TNFα did not affect cell proliferation, but high dose-exposure may induce cell death and senescence (26,27). Moreover, loss of matrix proteoglycans was revealed to be TNFα-dependent because TNFα blockade inhibited destruction of chondrocytes by TNFα. Therefore, anti-TNFα therapy may alleviate the destruction of matrix proteoglycans and hyaline cartilage in inflammatory arthritis.

When chondrocytes become hypertrophic, they express unique genes, such as *COL10* and *MMP13*; these genes indicate calcification or matrix mineralization (28,29). In line with these results, the present study showed that TNFα treatment upregulated *COL10* mRNA expression (data not shown). Collectively, these data suggested that TNFα stimulation of chondrocytes regulated MMP13 and COL10 expression, leading to chondrocyte hypertrophy, calcification or matrix mineralization.

TNFα plays a critical role in bone destruction in several types of inflammatory joint disease (30,31). Particularly in RA, focal bone loss occurs due to excessive bone resorption by osteoclasts. Moreover, bone formation by osteoblasts is impaired at the bone erosion site. Thus, anti-TNF therapies could delay the progression of bone destruction without bone erosion in patients with RA, leading to significant clinical improvement. TNFα in inflammatory arthritis has a destructive function in all of the components that comprise joints, namely osteoclasts, osteoblasts, and chondrocytes (32-34).

Blocking studies with an anti-TNFα agent in an arthritis model have shown that TNFα may drive cartilage and bone destruction (35,36). Treatment with TNFα inhibitors decreased inflammation, as well as bone and cartilage damage, in patients with RA (30,31). Although osteoclastogenesis is a more dominant mechanism in the bone erosion and cartilage destruction of inflammatory arthritis (8,34), there are fewer reports on the pathological mechanisms by which TNFα and its inhibitors influence physiological changes of hyaline cartilage in human knee joints.

OA is a degenerative joint disease caused by the degradation of hyaline cartilage. Cartilage loss in OA is associated with aging and mechanical stress, as well as inflammation. The primary cause of OA is aging and mechanical stress, which suppress the regenerative capacity of chondrocytes involved in extracellular matrix components comprising hyaline cartilage. Moreover, total knee replacement for OA is a significant health burden, and there are no therapeutic drugs available yet for the regeneration of hyaline cartilage (37,38). Thus, there is a significant unmet need for effective medical therapies for OA. The present study demonstrated that TNFα contributed to OA pathogenesis via NF-κB mechanisms affecting metabolic imbalance in chondrocytes, leading to structural joint damage (39). Similarly, it was recently demonstrated that patients with OA exhibited a notable response to anti-TNF therapy (40), indicating a pivotal role for TNFα in cartilage catabolism. Thus, TNF inhibitors may be a therapeutic option for patients with OA with severe inflammation or TNFα-driven cartilage destruction.

In conclusion, the present study showed that TNFα was associated with progressive destruction of hyaline cartilage in OA. Mechanistically, chondrocyte exposure to TNFα increased *MMP1*, *MMP3* and *MMP13* gene expression, causing degradation of ACAN and COL2 structural proteins in hyaline cartilage. During chondrogenic differentiation, TNFα stimulation decreased the expression of *COL2* and *ACAN*, but not *SOX9*, thereby decreasing relative chondrogenic size; these effects were reversed following treatment with anti-TNF agents. Therefore, treatment with anti-TNF therapy may relieve chondrocyte destruction in inflammatory arthritis.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JP, HP, YLL and SW performed all experiments. SJ, BN, JHY, and YGK designed the experiments. JP, HP, and SJ analyzed experimental data. JHY provided human knee joint samples. YGK and BN provided synovial fluid of patients with OA and RA. SJ and THK wrote the manuscript. JP, HP, and SJ confirm the authenticity of all the raw data. THK conceptualized and supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Studies involving human materials were performed in compliance with the Helsinki Declaration and were approved by the Ethics Committee of Hanyang University Hospital (approval no. IRB-2017-05-003) and Hanyang University Guri Hospital (approval no. 2018-07-024). All subjects provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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