Inhibition of HMGB1 suppresses inflammation and catabolism in temporomandibular joint osteoarthritis *via* NF-κB signaling pathway

Yan Yan Li,[1,2]* Ya Ping Feng,[1,2]* Li Liu,[3] Jin Ke,[1,2] Xing Long[1,2]

[1]The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) and Key Laboratory of Oral Biomedicine Ministry of Education, School and Hospital of Stomatology, Wuhan University, Wuhan
[2]Department of Oral and Maxillofacial Surgery, School & Hospital of Stomatology, Wuhan University, Wuhan
[3]Department of Prosthodontics, School of Stomatology Kunming Medical University, Kunming, China

**These authors contributed equally to this study*

ABSTRACT

HMGB1 is a highly conserved nuclear protein that is rapidly released into the extracellular environment during infection or tissue damage. In osteoarthritis, HMGB1 acts as a pro-inflammatory cytokine inducing a positive feedback loop for synovial inflammation and cartilage degradation. The aim of this study was to explore the role of HMGB1 in inflammation and catabolism of temporomandibular joint osteoarthritis (TMJOA) and whether inhibition of HMGB1 affects TMJOA. Human synovial fibroblasts were incubated with HMGB1, the expression of pro-inflammatory cytokines and catabolic mediators were measured by Western blot and ELISA. NF-κB signaling pathway involvement was studied by the NF-κB inhibitor and detected by Western blotting and immunofluorescence staining. TMJOA was induced by an injection of complete Freund's adjuvant (CFA) into anterosuperior compartment of rat's joint. An anti-HMGB1 antibody was used to assess the effect to HMGB1 in the synovium and cartilage of the CFA-induced TMJOA rats by hematoxylin and eosin, Safranin O, Masson trichrome staining, immunohistochemistry and immunofluorescence. HMGB1 markedly increased the production of MMP13, ADAMTS5, IL-1β and IL-6 through activating NF-κB signaling pathway in human synovial fibroblasts. *In vivo*, application of the HMGB1 neutralizing antibody effectively ameliorated the detrimental extent of TMJOA. Furthermore, the HMGB1 neutralizing antibody reduced the expression of NF-κB, pro-inflammatory cytokines and catabolic mediators in the synovium and cartilage of CFA-induced TMJOA rats. HMGB1 inhibition alleviates TMJOA by reducing synovial inflammation and cartilage catabolism possibly through suppressing the NF-κB signaling pathway and may become a therapeutic method against TMJOA.

Key words: HMGB1 neutralizing antibody; temporomandibular joint osteoarthritis; inflammation; catabolism; NF-κB.

Correspondence: Xing Long, The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) and Key Laboratory of Oral Biomedicine Ministry of Education, School and Hospital of Stomatology, Wuhan University, Wuhan 430079, China. Tel. +86.27.87686216 - Fax: +86.27.430079.
E-mail: longxing@whu.edu.cn

Contributions: YYL, YPF, contributed to the conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; LL, contributed to conception and design, critically revised the manuscript; JK, XL, contributed to conception and design, data analysis and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work, ensuring integrity and accuracy.

Conflict of interest: The authors declare that they have no competing interests, and all authors confirm accuracy.

Availability of data and materials: Any underlying research materials related to the study *(e.g.*, data samples, etc.) can be accessed.

Ethics approval: All experiments and sample collections were performed according to the standards of the Human Research Ethics Committee, School and Hospital of Stomatology, Wuhan University, Wuhan, China (protocol no. 2014LUNSHENZI24) and was conducted based on the recommendations of the Declaration of Helsinki.

Funding: This study was supported by the National Natural Science Foundation of China, grant no. 81771100 to X. Long.

Introduction

Temporomandibular joint osteoarthritis (TMJOA) is a chronic disease with the main pathological features of articular cartilage destruction, subchondral bone degeneration and synovitis of the whole joint.[1] Although, the etiology is not fully understood, it is a multifactorial disorder characterized by several cellular and molecular changes, such as an imbalance between cartilage anabolism and catabolism, infiltration of macrophages, synovial inflammation and activation of immune responses.[2]

HMGB1 is a highly conserved nuclear protein, binding to multiple cell surface receptors including Toll-like receptors (TLRs) TLR2 and TLR4, as well as the receptors for advanced glycosylation end products (RAGE) to stimulate the innate immune system and trigger inflammatory responses.[3] HMGB1 has been found at high levels extracellularly in inflamed joints, enhancing catabolic processes and inflammatory responses that contribute to disease progression. In knee osteoarthritis (OA), HMGB1 is overexpressed in synovium and synovial fluid of patients, and the HMGB1 levels in synovial fluid are associated with the severity of synovitis, pain, and daily activities in patients.[4] Studies show that HMGB1 and its receptor RAGE are expressed in OA cartilage and their expression correlates with the Osteoarthritis Research Society International (OARSI) histological score.[5] HMGB1 also synergizes with cytokines or fibronectin fragment to enhance the inflammation and upregulate the cartilage degrading enzymes.[6] Inflammation is a common accompaniment and contributes to the development of OA, characterized by a large number of pro-inflammatory mediators including cytokines including IL-1β and IL-6 and chemokines of innate immune response to joint injuries.[7,8]Pro-inflammatory cytokines such as IL-1β promote progressive degeneration of the articular cartilage through the production of matrix metalloproteinase (MMPs),[9] which degrade extracellular matrix components, including collagen and proteoglycans.[10] IL-1β may also induce other proinflammatory cytokines such as IL-6 and IL-8 expression in chondrocytes and synovial fibroblasts.[11,12] Moreover, there is increasing evidence that IL-6 plays an important role in the pathogenesis of OA, suggesting that high levels of IL-6 are linked to an increased risk of OA.[13]

Several strategies have been proposed to inhibit HMGB1, ranging from small natural or synthetic molecules, such as glycyrrhizin, box A, HMGB1-specific antibodies, as well as peptide.[14] Emerging findings reveal that glycyrrhizin inhibits HMGB1 expression and translocation and downregulates the expression of inflammatory cytokines by reducing HMGB1 chemo-attraction, which may have the therapeutic potential against HMGB1-mediated pathological diseases including neurological disorders.[15] As a special antagonist of HMGB1, box A has been reported to enhance anti-inflammatory activity when fused with the acidic C-terminal tail both *in vivo* and *in vitro*. Regarding the HMGB1-specific antibody, it binds to the DNA-binding surfaces of HMG boxes and this binding could prevent the interaction of other molecules, such as TLR4 and RAGE, and has been demonstrated beneficial effects in experimental models of ischemia and traumatic brain injury.[16,17]

In this study, we evaluated the regulation of HMGB1 on the pro-inflammatory and catabolic mediators and its downstream pathways in human TMJOA synovial fibroblasts. Furthermore, the HMGB1 neutralizing antibody was utilized to investigate the inhibition of HMGB1 ameliorates inflammation and catabolism both in the synovium and cartilage as well as the possible signaling pathway in CFA-induced TMJOA rats.

Materials and Methods

Samples collection and cell culture

The synovium specimens were obtained from 20 TMJOA patients [male:7, female:13; age (mean) 18-50 (28)] undergoing arthroplasty. Informed consents were obtained from all patients before surgeries. TMJOA was diagnosed by medical history, clinical manifestation and imagological examination according to the general revised classification criteria.[19] All experiments and sample collections were performed according to the standards of the Human Research Ethics Committee, School and Hospital of Stomatology, Wuhan University (protocol No. 2014LUNSHENZI24) and was conducted based on the recommendations of the Declaration of Helsinki.[20]

The synovium specimens were washed for three times with ice-cold PBS (Hyclone, USA) containing 100 U/mL penicillin (Hyclone, USA) and 100 mg/mL streptomycin (Hyclone, USA), and then cultured as 1~2 mm^3 explants in the Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) containing 20% fetal bovine serum (FBS, Hyclone, USA) in an incubator with 5% CO_2 at 37°C.[21] The culture medium was changed regularly, and the cells grew and fused to a density of about 80% for subculture. The synovial fibroblasts at the third passage were incubated with HMGB1 (0, 10, 50, 100 and 500 ng/mL; HMGBiotech, Milan, Italy) for 24 h or 100 ng/mL of HMGB1 for 0 ,3, 6, 9, 12, 24 and 48 h respectively. To investigate the role of NF-κB on HMGB1-induced inflammation and catabolism of synovial fibroblasts, PDTC (Sigma-Aldrich, St. Louis, MO, USA) was added 3 h in advance at a concentration of 50 μM, followed by stimulation with HMGB1 at the concentration of 100 ng/mL for further 24 h.

Western blotting

Protein was collected from different treated synovial fibroblasts. The concentrations of protein were measured with the BCA protein assay. As previously described,[22] equal amounts of each protein were loaded on a 10% SDS-PAGE gels, and then transferred electrically to PVDF membranes (Millipore, Burlington, MA, USA). After blocking for 2 h in 5% skim milk in TBST buffer at room temperature, the membranes were incubated with corresponding primary antibodies at 4°C for overnight, respectively, including rabbit anti-MMP13 (1:1000, 18165-1-AP; Proteintech, Wuhan, China), rabbit anti-ADAMTS5 (1:1000, ab41037, Abcam, Cambridge, MA, USA), rabbit anti-phospho-NF-κB p65 (1:1000, 3033; Cell Signaling Technology, Danvers, MA, USA) and NF-κB p65 (1:1000, 8242; Cell Signaling Technology). Then, PVDF membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody, and proteins were detected by a chemiluminescence ECL system (Advansta, San Jose, CA, USA) and quantitatively analyzed and normalized relative to β-actin using Image-J software.

ELISA

Supernatants were harvested from synovial fibroblasts subjected to different stimuli, and then protein levels including IL-6 and IL-1β were determined by the ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol.

Immunofluorescence staining for cells

The treated synovial fibroblasts were fixed with 4% paraformaldehyde at room temperature for 15 min and permeabilized with 0.25% Triton-X-100. Then, the synovial fibroblasts were blocked with PBSTx (Beyotime, Haimen, China) and incubated with the following antibody: rabbit anti-phospho-NF-κB p65 (1:1500, 3033, Cell Signaling Technology, USA) at 4°C for

overnight. After incubated with the Dylight 594, Goat Anti-Rabbit IgG (Abbkine, Wuhan, China) for 1 h at 37°C, the nuclei of cells were stained by 4'6-diamidino-2-phenylindole (DAPI, Beyotime). Finally, the treated synovial fibroblasts were imaged using confocal laser scanning microscopy.

A rat TMJOA model

Fifteen Sprague-Dawley rats (8 weeks in age, male, n=5 per group) were divided randomly into three groups: Control (without intervention), complete Freund's adjuvant (CFA) and CFA with anti HMGB1 antibody group. Briefly, 50 μL CFA (Sigma-Aldrich) was injected bilaterally into the anterosuperior compartment of TMJ of rats on the basis of Kameoka's anterosuperior puncture technique.[23] After 2 weeks, 5 rats previously injected with CFA were intraperitoneally injected with 100 μg/kg/day anti HMGB1 antibody (MAB1690-100; R&D System) for 7 days. The rats in each group were sacrificed respectively on day 21 (the schematic chart for the timeline of treating rats is shown in Figure 4A).[22]

Histological analysis

The TMJ samples were fixed with 4% paraformaldehyde, demineralized in 10% EDTA for 6 weeks, embedded in paraffin, and then cut into 5 μm sections parallel to the lateral surface of the condyle. The sections were dewaxed with xylene and rehydrated in gradient ethanol. Hematoxylin and eosin, Safranin O and Masson trichrome staining were carried out according to the manufacturer's protocol. The cartilage is divided into four layers including the fibrous layer, the proliferative layer, the pre hypertrophic layer and the hypertrophic layer and the thickness of the four layers measured is the cartilage thickness. The thickness of cartilage was determined from H&E staining on the basis of the previous methods.[24] The changes of proteoglycan in cartilage matrix were detected by Safranin O staining and the area of Safranin O staining (stained red) / cartilage area of the region was the percentage of proteoglycan in the region. Mineralized old trabecular bone and unmineralized new bone were stained red and blue respectively by Masson trichrome staining. The areas of unmineralized trabecular bone (stained blue) / subchondral bone of the region was the percentage of unmineralized bone areas. Three regions (anterior, middle and posterior condylar process) of each section were selected for measurement and the average was calculated for statistics, as previously described.[25]

Immunohistochemistry and immunofluorescence staining

After having been dewaxed and rehydrated, the sections were treated with citric acid buffer for heat-induced antigen retrieval and then incubated with serum to block unspecific ligations after endogenous peroxidase activity block. Subsequently, the sections were incubated with the following primary antibodies: ADAMTS5 (1:1000, ab41037; Abcam), MMP13 (1:300, 18165-1-AP, Proteintech), IL-6 (1:200, ab9243; Abcam), IL-1β (1:200, ab9722; Abcam) and the substitution of serum instead of primary antibody overnight at 4°C. Next day, the sections were incubated with secondary antibodies and avidin-biotin-peroxidase reagent successively. Positive staining was detected by utilizing chromogen diaminobenzidine (DAB). Finally, the sections were counterstained with hematoxylin. For measurement, five magnified visual fields (×10) of TMJ sections were randomly selected under an Olympus DP72 microscope by three inspectors, and then Image-Pro Plus 6.0 software was used to quantify the number of positive cells and total cells on selected areas. The sections for immunofluorescence were unspecific ligations blocked by serum following antigen-retrieving, and rabbit anti-phospho-NF-κB p65 (1:1500, 3033; Cell Signaling Technology) was incubated overnight at 4°C, then Dylight 594, Goat Anti-Rabbit IgG (Abbkine) was incubated at 37°C for 1 h, the nuclei were stained by DAPI. For the immunofluorescence analysis, Image-Pro Plus 6.0 software was used to quantify the number of positive cells and total cells marked with DAPI in selected synovium and cartilage area. Five fields of each section were counted and averaged by three observers independently according to the fluorescence intensity.

Statistical analysis

All data were presented as the mean with 95% confidence interval (CI). Data were calculated and visualized by using GraphPad Prism 8.0. A Shapiro-Wilk test was utilized to verify normality. Performing F-tests to determine whether variance of parametric or non-parametric populations were equal. One-way analysis of variance (ANOVA) was used among groups followed by Tukey's multiple comparison test to compare the mean between each group; otherwise, a non-parametric Mann-Whitney U test was performed.[26] A p-value <0.05 was considered significant statistically.

Results

HMGB1 regulates the production of MMP13, ADAMTS5, IL-1β and IL-6 from human TMJOA synovial fibroblasts

To determine whether extracellular HMGB1 can modulate pro-inflammatory cytokines and catabolic mediators' production in human TMJOA synovial fibroblasts, cells were incubated with gradient concentration of HMGB1 (0~500 ng/mL) for 24 h or 100 ng/mL of HMGB1 for 0, 3, 6, 9, 12, 24 and 48 h respectively and the levels of MMP13, ADAMTS5, IL-1β and IL-6 were detected by Western blotting. The expression of MMP13, ADAMTS5, IL-1β and IL-6 was up-regulated after HMGB1 treatment for 24 h in a dose-dependent manner. 100 ng/mL HMGB1 markedly increased the levels of MMP13 ($p<0.001$, 16.36-fold; CI: -1.013 to -0.7208), ADAMTS5 ($p<0.001$, 6.39-fold; CI: -0.6832 to -0.4796) and IL-6 ($p<0.001$, 2.59-fold; CI: -69.98 to -56.74) while IL-1β ($p<0.001$, 2.62-fold; CI: -77.26 to -37.23) production was substantially increased in 50 ng/mL HMGB1 group (Figure 1 A,C,E,G). However, there were no marked differences in the levels of MMP13 ($p=0.09$; CI: -0.2774 to 0.01443) and ADAMTS5 ($p=0.07$; CI: -0.1966 to 0.006945) in 10 ng/mL groups. The production of MMP13, ADAMTS5, IL-1β and IL-6 was then examined at different time points after HMGB1 treatment (100 ng/mL), and peaked at 24 h (MMP13: $p<0.001$, 17.03-fold - CI: -0.8955 to -0.6165; ADAMTS5: $p<0.001$, 13.64-fold - CI: -0.8249 to -0.4736; IL-1β: $p<0.001$, 2.53-fold - CI: -71.15 to -45.93; IL-6: $p<0.001$, 2.44-fold - CI: -64.61 to -48.49) (Figure 1 B,D,F,H). Therefore, the concentration of HMGB1 at 100 ng/mL for 24 h was utilized in the following studies.

HMGB1 induces the production of MMP13, ADAMTS5, IL-1β and IL-6 *via* activating NF-κB signaling pathway in TMJOA synovial fibroblasts.

Studies have shown that NF-κB pathway is intimately related to OA and a major regulator of pro-inflammatory and degradative genes in joints.[27] In this experiment, the effects of HMGB1 (0~500ng/mL) for 24 h or 100 ng/mL of HMGB1 for 0, 3, 6, 12, 24 and 48 h respectively on the activation of the NF-κB signaling pathway in TMJOA synovial fibroblasts were examined. The expression of phospho-NF-κB p65 was up-regulated after HMGB1 treatment in a time-dependent manner, and peaked at 12 h ($p<0.001$, 4.80-fold; CI: -0.7754 to -0.3029) (Figure 2A). Also, significant increase of phospho-NF-κB p65 ($P < 0.001$, 6.44-fold,

[CI: -0.9955 to -0.2415]) was detected by Western blotting in synovial fibroblasts treated with HMGB1 (100 ng/mL) for 24 h (Figure 2B). Further, immunofluorescence staining showed that the amount of nuclear NF-κB was markedly increased after exposure to HMGB1 at 100 ng/mL for 24 h while NF-κB is mainly located in the cytoplasm in control (Figure 2C). To further elucidate the role of NF-κB signaling, a silencing experiment was performed. The levels of MMP13 ($p<0.001$, 0.44-fold; CI: 0.4098 to 0.7519), ADAMTS5 ($p<0.001$, 0.28-fold; CI: 0.4724 to 0.6091), IL-1β ($p<0.001$, 0.46-fold; CI: 44.39 to 55.08) and IL-6 ($p<0.001$, 0.72-fold; CI: 17.19 to 33.20) were found to be significantly decreased in TMJOA synovial fibroblasts after treatment with the NF-κB inhibitor (50 μM), indicating that HMGB1 activates inflammation and catabolism of synovial fibroblasts through NF-κB signaling pathway (Figure 3).

Inhibition of HMGB1 decreases the expression of pro-inflammatory cytokines and catabolic mediators in rat synovium of TMJOA

To investigate the therapeutic potential of HMGB1 blockade in TMJOA, we intraperitoneally administered the HMGB1 neutralizing antibody to rats after CFA-injection and detected the expression of pro-inflammatory mediators (IL-1β, IL-6) and catabolic mediators (ADAMTS5, MMP13) in synovium of rats by immunohistochemistry to evaluate its effect on TMJOA. Application of the HMGB1 neutralizing antibody markedly reduced the production of MMP13 ($p<0.001$, 0.40-fold; CI: 35.92 to 57.81), ADAMTS5 ($p<0.001$, 0.78-fold; CI: -0.7266 to 17.70), IL-1β ($p<0.001$, 0.38-fold; CI: 40.11 to 56.49) and IL-6 ($p<0.001$, 0.48-fold; CI: 12.83 to 27.39) in the synovium compared with CFA group (Figure 4 A,B). These results indicated that the HMGB1 neutralizing antibody ameliorated rat TMJOA.

Inhibition of HMGB1 alleviates the damage of cartilage and subchondral bone in CFA-induced TMJOA

As shown in Figure 5, the CFA groups revealed that the thickness of the condylar cartilage became thinner ($p<0.001$, 0.57-fold; CI: -75.19 to -32.41), and the intensity and area of Safranin O staining ($p<0.001$, 0.88-fold; CI: -0.1471 to -0.05468) were greatly reduced in most cartilage areas, compared with control. Then we performed Masson trichrome staining and found that the unmineralized area of the subchondral bone in the CFA groups was larger ($p<0.001$, 0.42-fold; CI: 0.2150 to 0.2836). A week after intraperitoneal injection of anti-HMGB1 antibodies, the damage of cartilage and subchondral bone induced by CFA was significantly ameliorated (Figure 5 A-D). Immunohistochemical analysis further suggested the inhibitory effects of anti-HMGB1 antibodies on the expression of MMP13 ($p=0.04$, 0.71-fold; CI: 4.927 to 22.80), ADAMTS5 ($p=0.03$, 0.70-fold; CI: 5.763 to 23.02), IL-1β ($p<0.001$, 0.58-fold; CI: 20.33 to 38.75) and IL-6 ($p<0.001$, 0.49-fold; CI: 24.12 to 57.70) in the cartilage (Figure 5 E,F).

Inhibition of HMGB1 suppresses the NF-κB signaling pathway in CFA-induced TMJOA

Finally, we confirmed that the anti-HMGB1 antibody inhibited the NF-κB signaling pathway in TMJOA. The phospho-p65 levels were increased in the synovium ($p<0.001$, 3.99-fold; CI: 43.23 to 58.13) and cartilage ($p<0.001$, 4.62-fold; CI: 37.84 to 52.07) of CFA-induced TMJOA. Immunofluorescence staining showed that the percentages of phospho-p65-positive cells in the synovium ($p<0.001$, 0.44-fold; CI: 28.49 to 47.05) and cartilage ($p=0.002$, 0.75-fold; CI: 6.034 to 22.73) were significantly decreased in the CFA groups plus the anti-HMGB1 antibody rats compared to the CFA rats (Figure 6). Taken together, the anti-HMGB1 antibody may delay progression of TMJOA by reducing synovial inflammation and cartilage catabolism by suppressing the NF-κB signaling pathway (Figure 7).

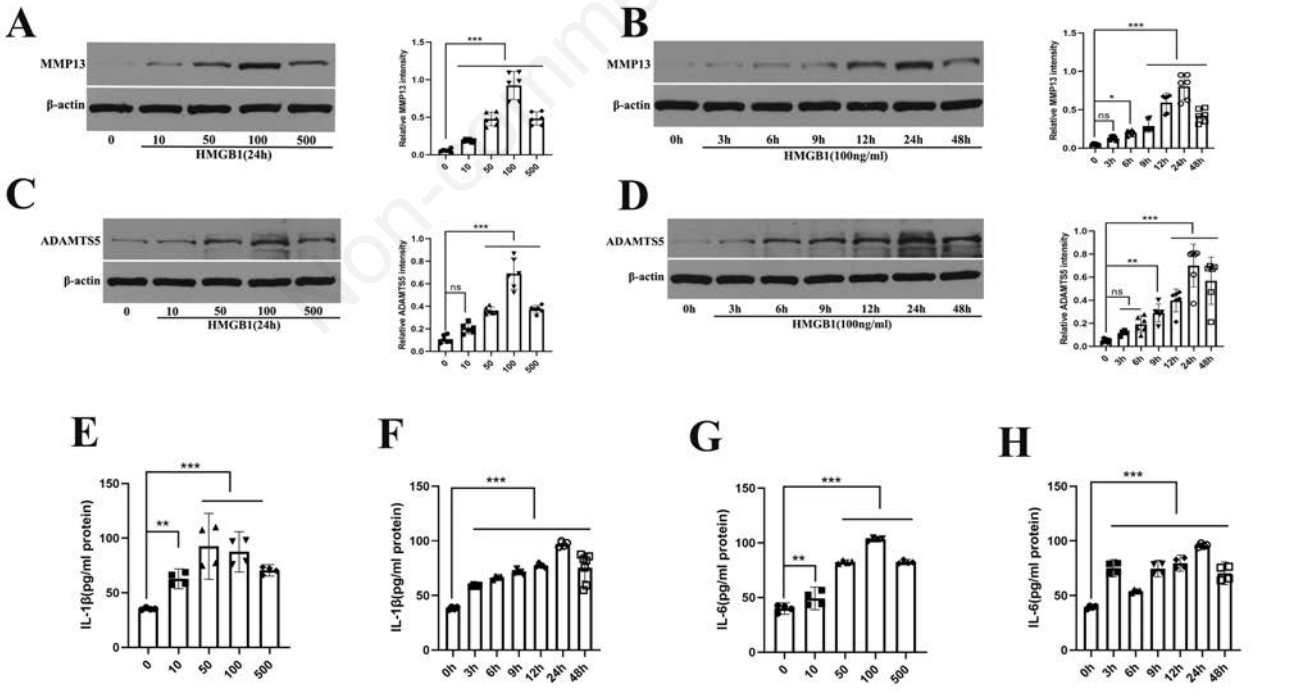

Figure 1. HMGB1 regulates the production of MMP13, ADAMTS5, IL-1β and IL-6 from TMJOA synovial fibroblasts. A,C,E,G) A dose-dependent augmentation of MMP13, ADAMTS5, IL-1β and IL-6 in synovial fibroblasts was induced by HMGB1 for 24 h. B,D,F,H) A time-dependent increase of MMP13, ADAMTS5, IL-1β and IL-6 in these cells was induced by 100 ng/mL of HMGB1. Western blotting assays were used to assay the relative expression levels; β-actin served as control; data are shown as the means with 95% CI and are analyzed by Mann-Whitney U test, n=6, *p< 0.05, **p<0.01, ***p<0.001.

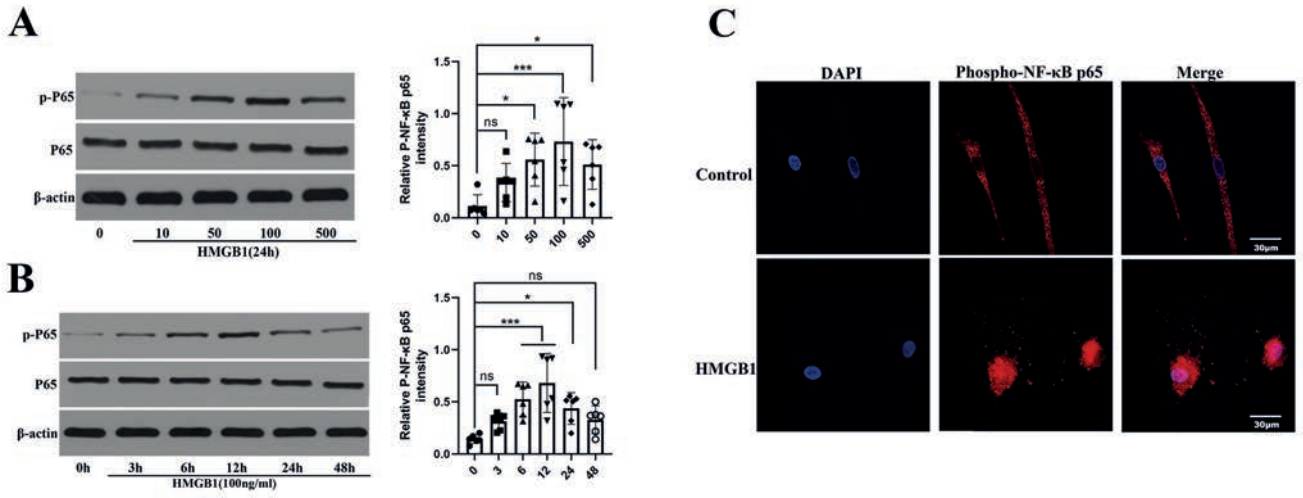

Figure 2. HMGB1 increases the phosphorylation level of the NF-κB subunit-P65 in TMJOA synovial fibroblasts. Western blotting analysis of phospho-p65, p65 in synovial fibroblasts cultured with different doses of HMGB1 for 24 h (A), or 100 ng/mL HMGB1 for various times (B); β-actin served as control; data are shown as the means with 95% CI and analyzed by Mann-Whitney U test, n=6, *p<0.05, **p<0.01, ***p<0.001. C) Immunofluorescence staining of phospho-p65 (red) in synovial fibroblasts treated with HMGB1. Scale bar: 30 μm.

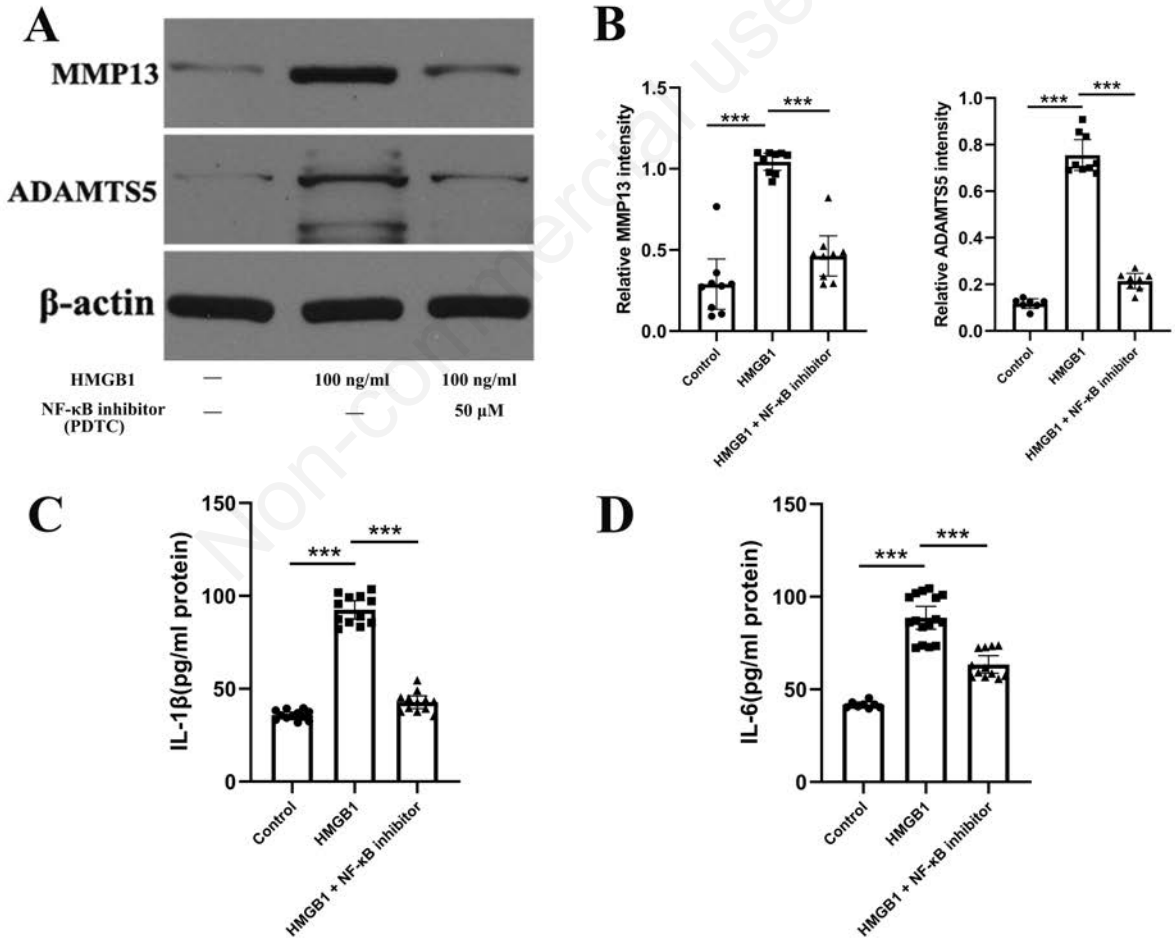

Figure 3. HMGB1 induces the production of MMP13, ADAMTS5, IL-1β and IL-6 through activating NF-κB signaling pathway in TMJOA synovial fibroblasts. A,B) Western blotting analysis of expression of MMP13 and ADAMTS5 in synovial fibroblasts treated with HMGB1 (100 ng/mL) or NF-κB inhibitor (50 μM). C,D) ELISA assay was to assay the secretion of IL-1β and IL-6 in the supernatants harvested from synovial fibroblasts treated with HMGB1 or NF-κB inhibitor; data are shown as the means with 95% CI and are analyzed using one-way analysis of variance (ANOVA), n=9, ***p<0.001.

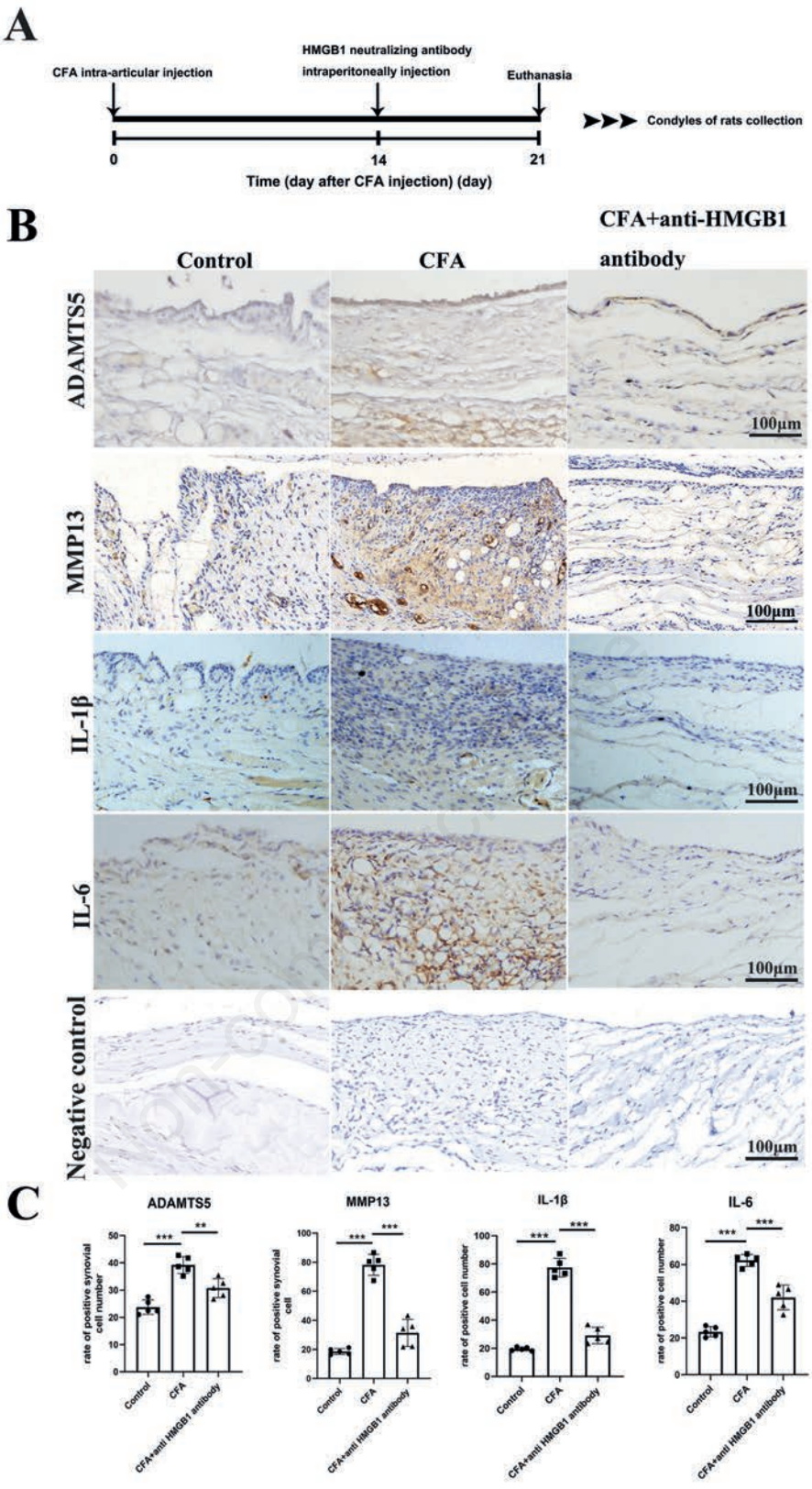

Figure 4. Inhibition of HMGB1 decreases the expression of pro-inflammatory cytokines and catabolic mediators in rat synovium of CFA induced TMJOA. A) The schematic chart for the timeline of treating rats. B) The expression of ADAMTS5, MMP13, IL-1β, IL-6 and negative controls was detected by immunohistochemistry in the synovium of CFA groups, CFA with anti-HMGB1 antibody treatment groups at day 21, or in the control synovium; scale bar: 100 μm. C) Quantitative analysis of ADAMTS5, MMP13, IL-1β and IL-6 in the synovium of rats; data are shown as the means with 95% CI and analyzed by one-way analysis of variance (ANOVA), n=5, *p<0.05, **p<0.01, ***p<0.001.

Discussion

Previous study has demonstrated that HMGB1 effectively upregulated the production of VEGF and HIF-1α in TMJOA synovial fibroblasts and regulated synovial angiogenesis.[22] It is generally believed that angiogenesis promotes the release of inflammatory mediators, leading to imbalance between anabolism and catabolism of the surrounding tissues.[28] When the equilibrium of chondrocyte metabolic activity is disturbed, the synthesis of cartilage matrix is inadequate, the catabolism increases, resulting in the destruction of the structural and functional integrity of cartilage.[29] Herein, we demonstrated that increasing HMGB1 levels in human synovial fibroblasts alone was sufficient to enhance the expression of TMJOA-associated catabolic and inflammatory proteins, particularly at the concentration of 100 ng/mL. It showed that a high concentration of HMGB1 slightly inhibited the growth of synovial fibroblast, possibly because of the cytotoxicity of high concentration of HMGB1 to synovial fibroblasts. It is well-known that synovitis is a common symptom in most patients with OA, even in the early phase of the disease.[30] Several lines of evidence support that the synovial fibroblasts produce catabolic mediators, such as MMP-1, MMP-13, and ADAMTS-5, and pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α that contribute to cartilage degradation in OA.[31,32] In OA, HMGB1 plays an important role as a pro-inflammatory cytokine inducing positive-feedback loops of synovial fibroblasts reactivation, inflammation and cartilage degradation through the release of TNF-α, IL-1β, IL-6, IL-8 and MMPs.[4] Specifically, these pro-inflammatory factors such as IL-1β are produced by the synovial membrane and released into the synovial fluid, then diffused into the cartilage, which exert catabolic effects on the chondrocyte metabolism by decreasing collagen and aggrecan synthesis and increasing the production of MMPs.[33]

Considering that HMGB1 plays vital roles during development of catabolic and inflammatory responses in synovial fibroblasts of TMJOA, we applied an anti-HMGB1 antibody in CFA-induced TMJOA rats. The articular injection of CFA is a repeatable and easy method to establish the animal model of TMJOA. In addition, previous studies have shown that synovial cells were apparently hyperplasic in the inflammatory synovium and there were time-dependent degeneration manners of TMJ cartilage and subchondral bone after CFA injection of rat model.[24] In this study, the intraperitoneal injection of an anti-HMGB1 antibody markedly reduced the expression of pro-inflammatory mediators, suppressed the upregulation of catabolic mediators of synovium and cartilage,

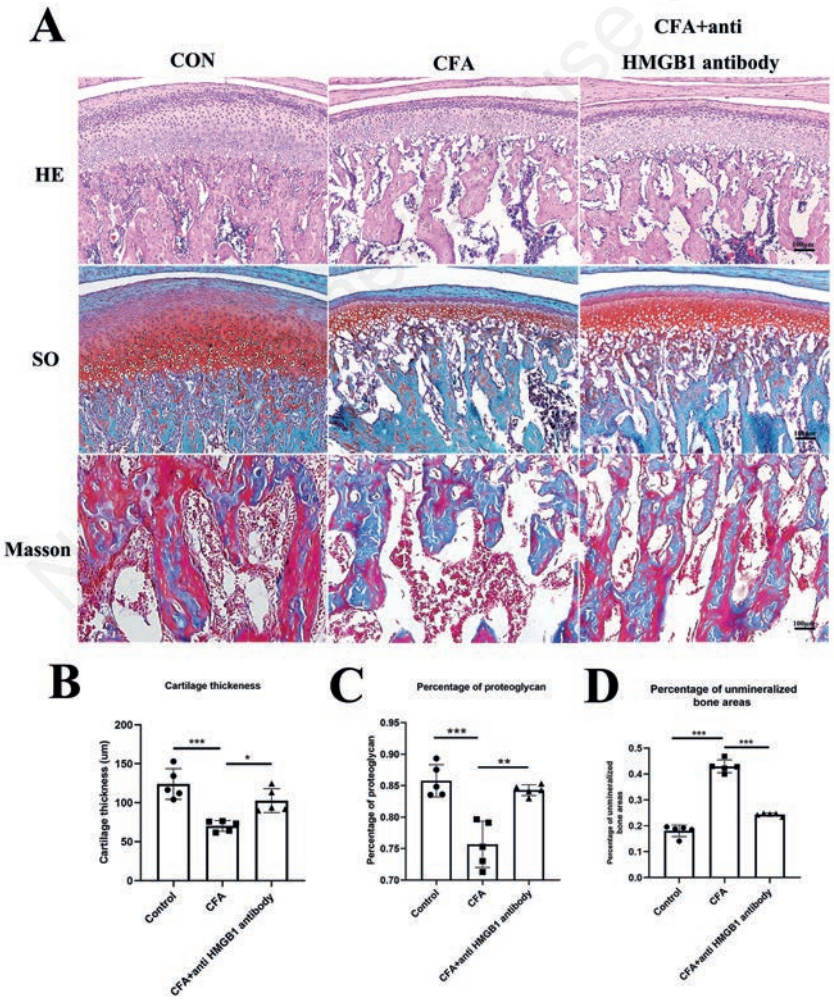

Figure 5. Inhibition of HMGB1 alleviates the damage of cartilage and subchondral bone in CFA induced TMJOA. A) H&E, Safranin O and Masson trichrome staining of rat models at day 21; scale bar: 100 μm. B-D) Analysis of the condylar cartilage thickness, the Safranin O positive areas and unmineralized bone areas; data are shown as the means with 95% CI and analyzed by one-way analysis of variance (ANOVA), n=5, *p<0.05, **p<0.01, ***p<0.001.

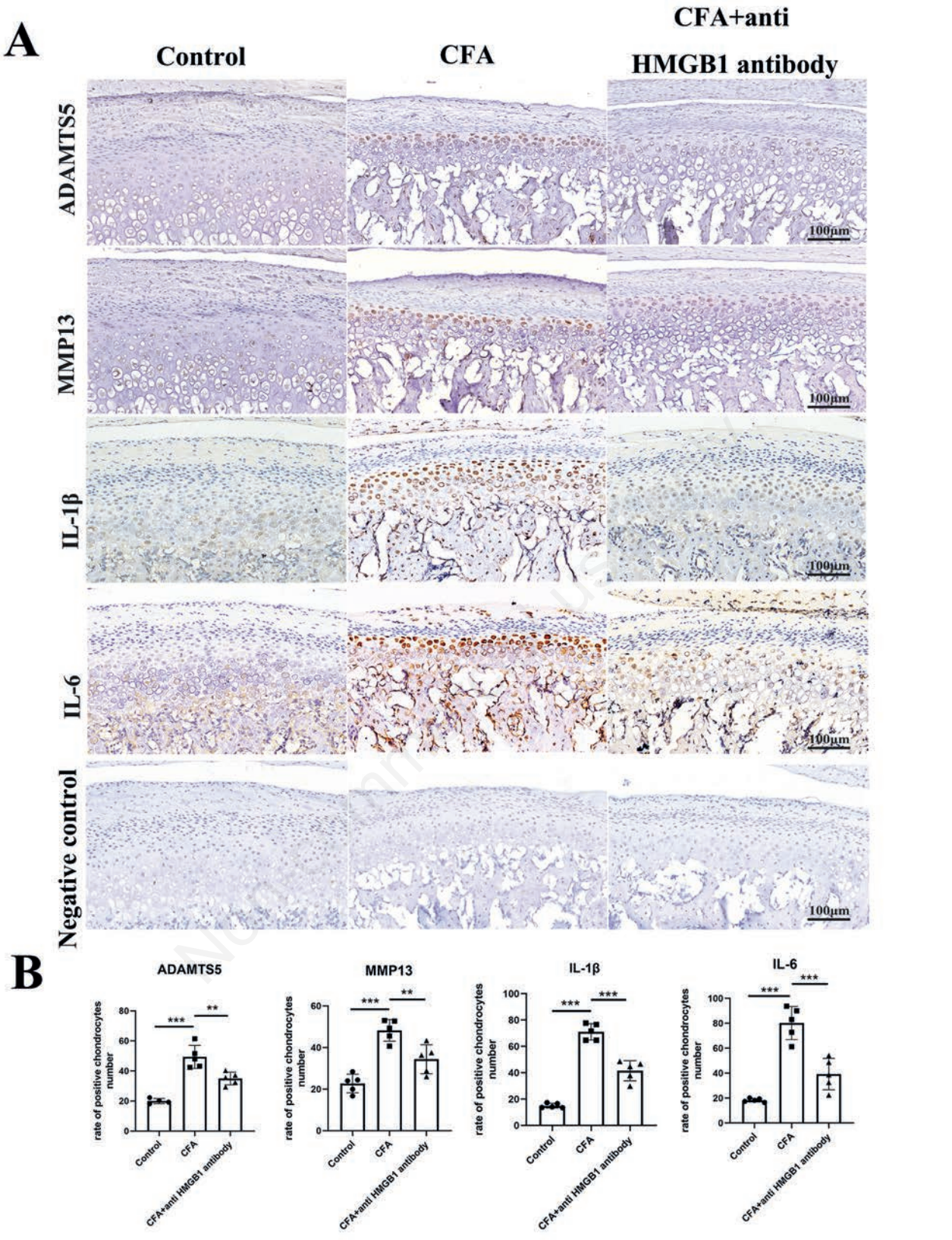

Figure 6. Inhibition of HMGB1 decreases the expression of pro-inflammatory cytokines and catabolic mediators in rat cartilage of CFA induced TMJOA. A) The expression of ADAMTS5, MMP13, IL-1β, IL-6 and negative controls was detected by immunohistochemistry in the cartilage of CFA groups, CFA with anti-HMGB1 antibody treatment groups at day 21, or in the control cartilage; scale bar: 100 μm. B) Rate of ADAMTS5, MMP13, IL-1β and IL-6 positive cells in the cartilage was markedly increased in the CFA groups, while markedly inhibited after treatment with anti-HMGB1 antibody; data are shown as the means with 95% CI and analyzed by one-way analysis of variance (ANOVA), n=5, *p<0.05, **p<0.01, ***p<0.001.

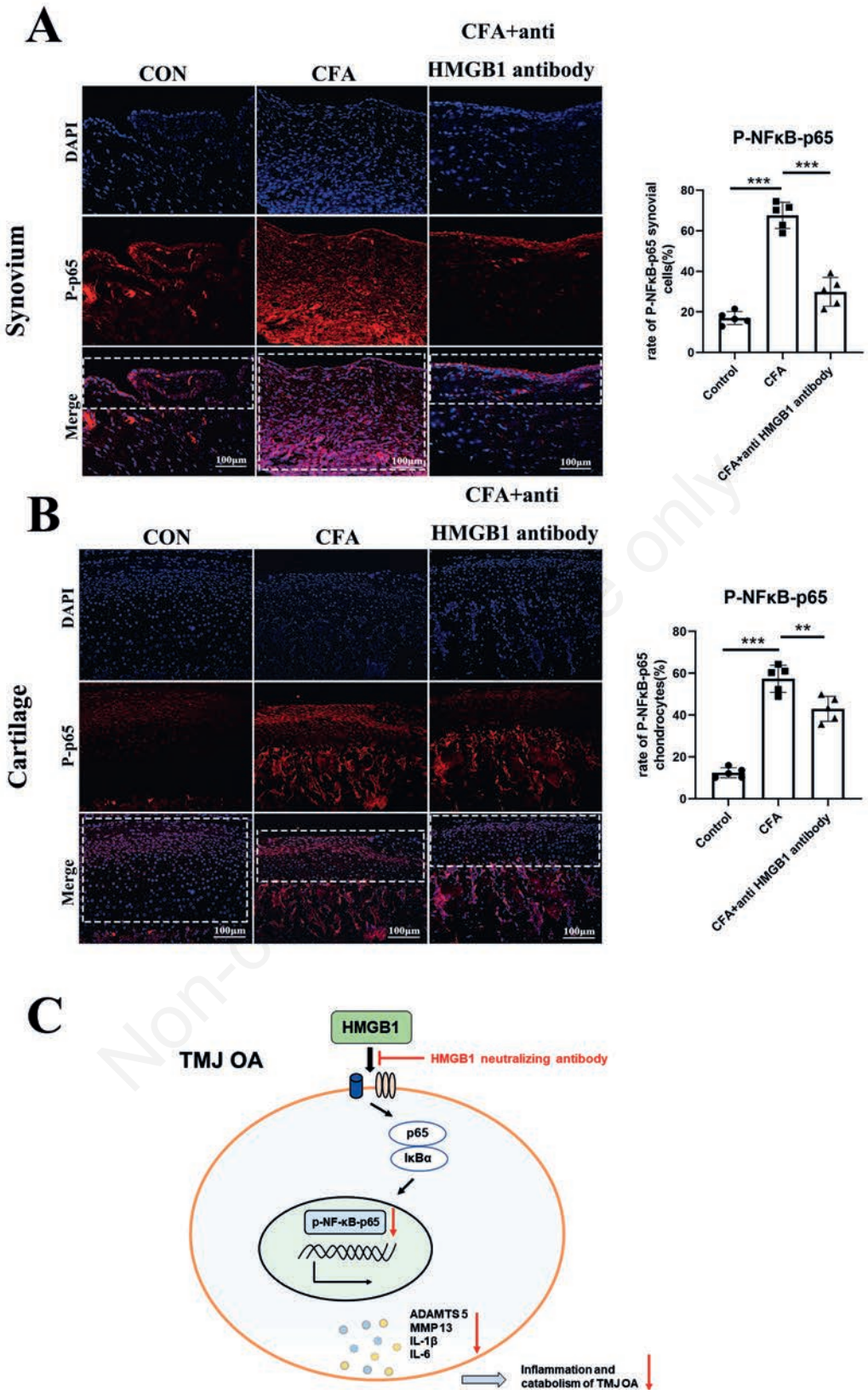

Figure 7. Inhibition of HMGB1 suppresses the NF-κB signaling pathway *in vivo*. Expression of phospho-p65 in the synovium (A) and cartilage (B) of CFA groups, CFA with anti-HMGB1 antibody treatment groups at day 21 were detected by IF staining, or in the control groups; data are shown as the means with 95% CI and analyzed by one-way analysis of variance (ANOVA), n=5, *p<0.05, **p<0.01, ***p<0.001; scale bar: 100 μm. C) Schematic diagram of inhibition of HMGB1 suppresses inflammation and catabolism in temporomandibular joint osteoarthritis *via* NF-κB signaling pathway.

and attenuated synovium inflammation, cartilage degradation and subchondral bone destruction in the TMJOA model. Some studies have shown that the inhibition of HMGB1 with a neutralizing anti-HMGB1 antibody can afford a therapeutic effect of inflammatory disease.[34] Short term treatment with polyclonal anti-HMGB1 antibodies was proved to reduce destruction to the joints and improved arthritic symptoms of collagen induced arthritis (CIA) rodents *via* interacting with the numerous epitopes of the extracellular HMGB1,[35] and early HMGB1 blockade ameliorates cartilage damage in experimental OA.[36] Actually, cartilage and synovium can communicate through synovial fluid and share some inflammatory signaling pathways, and it is widely accepted by researchers that degraded cartilage fragments fall into the joint and activate synovial fibroblasts to produce inflammatory mediators, which can not only cause cartilage degradation but also induce synovial angiogenesis and increase the synthesis of inflammatory cytokines and MMPs by synovial fibroblasts themselves (vicious circle).[37] It should be noted that they are two different types of tissues and may have different responses to the same stimuli in OA, while HMGB1 has similar OA-responsive expressions in cartilage and synovium, and inhibition of HMGB1 protects and repairs both cartilage and synovium, which should be a potential target for OA treatment.[4]

Furthermore, we investigated a mechanism of HMGB1 inhibition during TMJOA. HMGB1 has three distinct domains: boxes A and B and acid C-terminal tails, recognized by RAGE and TLR2/4. In general, HMGB1 antagonists competitively interact with the receptors including RAGE to inhibit the production of pro-inflammatory cytokines.[14] Some studies have found that the administration of anti-HMGB1 antibody can promote the improvement of tissue damage in a murine model of severe hemolytic uremia, and it is believed that the anti-HMGB1 antibody recognizes the C-terminal sequence (Box B) of HMGB1, which locates near the RAGE binding region, and suppresses the release of IL-6 and TNF-a.[38] In Jun's research, they treated subarachnoid hemorrhage (SAH) rats with the same anti-HMGB1 antibody that we utilized, which inhibited the translocation and release of HMGB1 in smooth muscle cells and the activity in the HMGB1-induced TLR4 pathway, and significantly decreased the expression of TLR4, IL-6, TNF-α and inducible nitric oxide synthase (iNOS) in basilar artery.[39] The binding of HMGB1 to RAGE and TLR2/4 promotes the activation of the NF-κB signaling pathway in inflammation.[40] NF-κB is a dimer composed of two proteins, p65 and IκBα, under resting conditions. Phosphorylation and subsequent degradation of IκBα lead to p65 phosphorylation, activation, and translocation from the cytoplasm to the nucleus, where it facilitates the transcription of its target genes, including many inflammation-related genes.[41] NF-κB signaling pathway is one of the major catabolic signaling pathways regulating cartilage homeostasis and OA progression, playing a critical role in the regulation of inflammatory mediators associated with OA.[42] NF-κB inactivation reduces the expression of downstream pro-inflammatory cytokines, matrix-degrading proteinases, catabolic cytokines and chemokines, which leads to the regulated accumulation and remodeling of ECM proteins and contributes to the control of synovial inflammation and cartilage degradation.[27,43] The antagonist of HMGB1 effectively suppressed the IL-1β-induced phospho-p65 phosphorylation and reduced the expression of MMPs and inflammatory mediators in human chondrocytes *via* HMGB1/TLR4/NF-κB pathway.[44] In the present study, immunofluorescence data clearly showed that HMGB1 induced p65 nuclear accumulation in synovial fibroblasts and inhibition of NF-κB significantly decreased the levels of MMP13, ADAMTS5, IL-1β and IL-6 in TMJOA synovial fibroblasts. Additionally, the application of an anti-HMGB1 antibody decreased the levels of phospho-p65, pro-inflammatory cytokines and catabolic mediators in the synovium and cartilage *in vivo*. Also, the expression of HMGB1, RAGE, TLR2 and TLR4 were up-regulated in OA cartilage,[45] we speculated that the inhibition of HMGB1 blocked the binding of HMGB1 to RAGE and TLR2/4 to quench the NF-κB activity and suppressed inflammation and catabolism in TMJOA.

Collectively, the results demonstrated that HMGB1 increased MMP13, ADAMTS5, IL-1β and IL-6 production in human synovial fibroblasts and blockade of HMGB1 reduced inflammatory responses and catabolism in CFA-induced TMJOA rats, possibly *via* deactivating the NF-κB signaling pathway, which provided a potential treatment strategy for TMJOA.

References

1. Wang XD, Zhang JN, Gan YH, Zhou YH. Current understanding of pathogenesis and treatment of TMJ osteoarthritis. J Dent Res 2015;94:666-73.
2. Robinson W, Lepus C, Wang Q, Raghu H, Mao R, Lindstrom T, et al. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. Nat Rev Rheumatol 2016;12:580-92.
3. Andersson U, Yang H, Harris H. High-mobility group box 1 protein (HMGB1) operates as an alarmin outside as well as inside cells. Sem Immunol 2018;38:40-8.
4. Nefla M, Holzinger D, Berenbaum F, Jacques C. The danger from within: alarmins in arthritis. Nat Rev Rheumatol 2016;12:669-83.
5. Terada C, Yoshida A, Nasu Y, Mori S, Tomono Y, Tanaka M, et al. Gene expression and localization of high-mobility group box chromosomal protein-1 (HMGB-1) in human osteoarthritic cartilage. Acta Med Okayama 2011;65:369-77.
6. Ding L, Buckwalter J, Martin J. DAMPs Synergize with cytokines or fibronectin fragment on inducing chondrolysis but lose effect when acting alone. Mediators Inflamm 2017;2017:2642549.
7. Li X, Li Y, Yang X, Liao R, Chen L, Guo Q, et al. PR11-364P22.2/ATF3 protein interaction mediates IL-1-induced catabolic effects in cartilage tissue and chondrocytes. J Cell Mol Med 2021;25:6188-202.
8. Liu-Bryan R, Terkeltaub R. Emerging regulators of the inflammatory process in osteoarthritis. Nat Rev Rheumatol 2015;11:35-44.
9. Goldring M, Otero M. Inflammation in osteoarthritis. Curr Opin Rheumatol 2011;23:471-8.
10. Loreto C, Filetti V, Almeida L, La Rosa G, Leonardi R, Grippaudo C, et al. MMP-7 and MMP-9 are overexpressed in the synovial tissue from severe temporomandibular joint dysfunction. Eur J Histochem 2020;64:3113.
11. Alquraini A, Jamal M, Zhang L, Schmidt T, Jay G, Elsaid K. The autocrine role of proteoglycan-4 (PRG4) in modulating osteoarthritic synoviocyte proliferation and expression of matrix degrading enzymes. Arthritis Res Ther 2017;19:89.
12. de Seny D, Cobraiville G, Charlier E, Neuville S, Esser N, Malaise D, et al. Acute-phase serum amyloid a in osteoarthritis: regulatory mechanism and proinflammatory properties. PloS One 2013;8:e66769.
13. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier J, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol 2011;7:33-42.
14. Musumeci D, Roviello GN, Montesarchio D. An overview on HMGB1 inhibitors as potential therapeutic agents in HMGB1-related pathologies. Pharmacol Ther 2014;141:347-57.
15. Paudel YN, Angelopoulou E, Semple B, Piperi C, Othman I, Shaikh MF. Potential neuroprotective effect of the HMGB1 inhibitor glycyrrhizin in neurological disorders. ACS Chem

Neurosci 2020;11:485-500.
16. Yang H, Wang H, Andersson U. Targeting inflammation driven by HMGB1. Front Immunol 2020;11:484.
17. Pisetsky DS, Erlandsson-Harris H, Andersson U. High-mobility group box protein 1 (HMGB1): an alarmin mediating the pathogenesis of rheumatic disease. Arthritis Res Ther 2008;10:209.
18. Feng Y, Fang W, Li C, Guo H, Li Y, Long X. The expression of high-mobility group box protein-1 in temporomandibular joint osteoarthritis with disc perforation. J Oral Pathol Med 2016;45:148-52.
19. de Souza R, Lovato da Silva C, Nasser M, Fedorowicz Z, Al-Muharraqi M. Interventions for the management of temporomandibular joint osteoarthritis. Cochrane Database Syst Rev 2012(4):CD007261.
20. Shrestha B, Dunn L. The Declaration of Helsinki on medical research involving human subjects: A review of seventh revision. J Nepal Health Res Counc 2020;17:548-52.
21. Nagai H, Miyamoto Y, Nakata A, Hatakeyama S, Iwanami Y, Fukuda M. Isolation and characterization of synovial cells from the human temporomandibular joint. J Oral Pathol Med 2006;35:104-10.
22. Feng Y, Hu S, Liu L, Ke J, Long X. HMGB1 contributes to osteoarthritis of temporomandibular joint by inducing synovial angiogenesis. J Oral Rehabil 2021;48:551-9.
23. Kameoka S, Matsumoto K, Kai Y, Yonehara Y, Arai Y, Honda K. Establishment of temporomandibular joint puncture technique in rats using in vivo micro-computed tomography (R_mCT®). Dentomaxillofac Radiol 2010;39:441-5.
24. Xu L, Guo H, Li C, Xu J, Fang W, Long X. A time-dependent degeneration manner of condyle in rat CFA-induced inflamed TMJ. Am J Transl Res 2016;8:556-67.
25. Liu X, Cai HX, Cao PY, Feng Y, Jiang HH, Liu L, et al. TLR4 contributes to the damage of cartilage and subchondral bone in discectomy-induced TMJOA mice. J Cell Mol Med 2020;24:11489-99.
26. Bland J, Altman D. Multiple significance tests: the Bonferroni method. BMJ 1995;310:170.
27. Rigoglou S, Papavassiliou A. The NF-κB signalling pathway in osteoarthritis. Int J Biochem Cell Biol 2013;45:2580-4.
28. Risbud MV, Shapiro IM. Role of cytokines in intervertebral disc degeneration: pain and disc content. Nat Rev Rheumatol 2014;10:44-56.
29. Musumeci G, Castrogiovanni P, Mazzone V, Szychlinska M, Castorina S, Loreto C. Histochemistry as a unique approach for investigating normal and osteoarthritic cartilage. Eur J Histochem 2014;58:2371.
30. Mathiessen A, Conaghan P. Synovitis in osteoarthritis: current understanding with therapeutic implications. Arthritis Res Ther 2017;19:18.
31. Abramson S, Attur M. Developments in the scientific understanding of osteoarthritis. Arthritis Res Ther 2009;11:227.
32. Castrogiovanni P, Di Rosa M, Ravalli S, Castorina A, Guglielmino C, Imbesi R, et al. Moderate physical activity as a prevention method for knee osteoarthritis and the role of synoviocytes as biological key. Int J Mol Sci 2019;20:511.
33. Wang P, Liu C, Yang X, Zhou Y, Wei X, Ji Q, et al. Effects of low-level laser therapy on joint pain, synovitis, anabolic, and catabolic factors in a progressive osteoarthritis rabbit model. Lasers Med Sci 2014;29:1875-85.
34. Sasaki T, Liu K, Agari T, Yasuhara T, Morimoto J, Okazaki M, et al. Anti-high mobility group box 1 antibody exerts neuroprotection in a rat model of Parkinson's disease. ExpNeurol 2016:220-31.
35. Schierbeck H, Lundbäck P, Palmblad K, Klevenvall L, Erlandsson-Harris H, Andersson U, et al. Monoclonal anti-HMGB1 (high mobility group box chromosomal protein 1) antibody protection in two experimental arthritis models. Mol Med 2011;17:1039-44.
36. Aulin C, Lassacher T, Palmblad K, Erlandsson Harris H. Early stage blockade of the alarmin HMGB1 reduces cartilage destruction in experimental OA. Osteoarthritis Cartilage 2020;28:698-707.
37. Berenbaum F. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). Osteoarthritis Cartilage 2013;21:16-21.
38. Maeda R, Kawasaki Y, Kume Y, Go H, Suyama K, Hosoya M. Involvement of high mobility group box 1 in the pathogenesis of severe hemolytic uremic syndrome in a murine model. Am J Physiol Renal Physiol 2019;317:F1420-9.
39. Haruma J, Teshigawara K, Hishikawa T, Wang D, Liu K, Wake H, et al. Anti-high mobility group box-1 (HMGB1) antibody attenuates delayed cerebral vasospasm and brain injury after subarachnoid hemorrhage in rats. Sci Rep 2016;6:37755.
40. Andersson U, Erlandsson-Harris H. HMGB1 is a potent trigger of arthritis. J Intern Med 2004;255:344-50.
41. Lepetsos P, Papavassiliou K, Papavassiliou A. Redox and NF-κB signaling in osteoarthritis. Free Radic Biol Med 2019;132:90-100.
42. Kobayashi H, Chang S, Mori D, Itoh S, Hirata M, Hosaka Y, et al. Biphasic regulation of chondrocytes by Rela through induction of anti-apoptotic and catabolic target genes. Nat Commun 2016;7:13336.
43. Marcu K, Otero M, Olivotto E, Borzi R, Goldring M. NF-kappaB signaling: multiple angles to target OA. Curr Drug Targets 2010;11:599-613.
44. Fu Y, Lei J, Zhuang Y, Zhang K, Lu D. Overexpression of HMGB1 A-box reduced IL-1β-induced MMP expression and the production of inflammatory mediators in human chondrocytes. Exp Cell Res 2016;349:184-90.
45. Lin SS, Yuan LJ, Niu CC, Tu YK, Yang CY, Ueng SWN. Hyperbaric oxygen inhibits the HMGB1/RAGE signaling pathway by upregulating Mir-107 expression in human osteoarthritic chondrocytes. Osteoarthritis Cartilage 2019;27:1372-81.

Received for publication: 2 November 2021. Accepted for publication: 24 May 2022.

European Journal of Histochemistry 2022; 66:3357
doi:10.4081/ejh.2022.3357