BJR

■ ARTHRITIS

Meniscus cell lysate induces mitochondrial dysfunction of fibroblast-like synoviocytes via upregulating ANT3 in osteoarthritis

ANT3 IS A POTENTIALLY IMPORTANT NOVEL MEDIATOR AND DRUG TARGET IN OSTEOARTHRITIS

X. Du,
Z. Jiang,
G. Fang,
R. Liu,
X. Wen,
Y. Wu,
S. Hu,
Z. Zhang

From The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

Aims

This study aimed to investigate the role and mechanism of meniscal cell lysate (MCL) in fibroblast-like synoviocytes (FLSs) and osteoarthritis (OA).

Methods

Meniscus and synovial tissue were collected from 14 patients with and without OA. MCL and FLS proteins were extracted and analyzed by liquid chromatography–mass spectrometry (LC–MS). The roles of MCL and adenine nucleotide translocase 3 (ANT3) in FLSs were examined by enzyme-linked immunosorbent assay (ELISA), flow cytometry, immunofluorescence, and transmission electron microscopy. Histological analysis was performed to determine ANT3 expression levels in a male mouse model.

Results

We discovered for the first time that MCL was substantially enriched in the synovial fluid of OA patients and promoted the release of inflammatory cytokines from FLSs through MCL phagocytosis. Through LC–MS, ANT3 was identified and determined to be significantly upregulated in MCL and OA-FLSs, corresponding to impaired mitochondrial function and cell viability in OA-FLSs. Mitochondrial homeostasis was restored by ANT3 suppression, thereby alleviating synovial inflammation. Furthermore, elevated ANT3 levels inhibited ERK phosphorylation. Specifically, silencing ANT3 prevented inhibition of ERK phosphorylation and significantly reduced the elevation of reactive oxygen species (ROS) and JC1 membrane potential in MCL-induced synovial inflammation.

Conclusion

This study revealed the important roles of MCL and ANT3 in FLS mitochondria. Silencing ANT3 rescued ERK phosphorylation, thereby restoring mitochondrial homeostasis in FLSs and alleviating synovitis and OA development, offering a potential target for treating synovitis and preventing early-stage OA.

Correspondence should be sent to Zhiqi Zhang; email: zhzhiqi@mail.sysu.edu.cn

Article focus

■ This study investigated the role of meniscal cell lysate (MCL) in the development of osteoarthritis (OA) synovitis and the underlying mechanisms.

Key messages
- Following MCL treatment, compared with normal fibroblast-like synoviocytes (FLSs) the levels of relevant inflammatory factors were significantly elevated, and cell viability was simultaneously impaired.
- MCL stimulated adenine nucleotide translocase 3 (ANT3) expression in FLSs, thereby inhibiting ERK phosphorylation.
- ANT3 knockdown prevented inhibition of ERK phosphorylation and significantly reduced the elevation of reactive oxygen species (ROS) and JC1 membrane potential in MCL-induced synovial inflammation.

Strengths and limitations
- These findings may provide potential targets for the treatment of synovitis and prevention of early OA.
- However, more in-depth research is required to uncover which cytokines in MCL lead to increased synovitis, and animal studies of ANT3 knockout are necessary to establish a better understanding of the pathogenesis of OA synovitis.

Introduction
Osteoarthritis (OA) is a common musculoskeletal morbidity and one of the leading causes of disability worldwide.[1] Currently, OA is considered a systemic disease that can affect multiple tissues in joints, especially synovium.[2,3] Resident fibroblast-like and macrophage-like synoviocytes (FLSs and MLSs) release proinflammatory factors that induce positive feedback of cytokine and catabolic enzyme secretion by synoviocytes and chondrocytes, leading to pannus-like thickening, increased cellularity, and cartilage degeneration.[4] Therefore, patients with even mild synovitis can suffer excessive pain and stiffness.[5-7] Hence, it is necessary to elucidate the mechanism of synovial inflammation.

OA is also associated with physical damage to other tissues, including cartilage, meniscus, and ligaments. A recent study reported that cartilage damage unleashed chondrocyte cell lysate, which might contribute to joint inflammation.[8] However, aside from cartilage damage, meniscal lesions represent one of the most frequently occurring intra-articular knee injuries. Many studies have suggested that meniscal lesions are more likely to lead to pain and OA,[9] but it has not been reported whether meniscal cell lysate (MCL) leads to the exacerbation of OA.[8,10] There is a need for a greater understanding of the role of synovitis in patients with meniscal tears and OA.[11] We manufactured MCL according to the method of producing chondrocyte lysates to study whether MCL plays a key role in the progression of synovitis and knee OA.

Mitochondria, which are membrane-enclosed organelles, play a crucial role in cellular metabolism. Some studies have shown that mitochondrial dysfunction is a hallmark of ageing and contributes to the pathophysiological process of OA. Cell death originating from mitochondria is often mediated by various mitochondrial channels and regulators, including adenine nucleotide translocase (ANT), mitochondrial Ca^{2+} uniporter (MCU), and cyclophilin D (CypD), and can be caused by various metabolic stresses including deregulated mitochondrial redox or Ca^{2+} flux.[12,13] ANTs are the most abundant proteins in the mitochondrial inner membrane.[14] In humans there are four closely related isoforms (ANT1, 2, 3, and 4) sharing 60% to 80% identical sequences. ANT1, ANT2, and ANT4 are expressed in a tissue-specific manner, whereas ANT3 is ubiquitously expressed.[15,16] ANT3 plays a role in translocating adenosine diphosphate (ADP) from the cytoplasm into the mitochondrial matrix and adenosine triphosphate (ATP) from the mitochondrial matrix into the cytoplasm.[17] Overexpression of ANT1 or ANT3 has been reported to induce apoptosis with a concomitant decrease in mitochondrial inner membrane potential, whereas the overexpression of ANT2 had no effect on cell death.[18]

In this study, we observed that MCL can stimulate the synovial inflammatory response via synoviocyte upregulation of ANT3 expression. ANT3 simultaneously induces mitochondrial dysfunction in FLSs through the ERK pathway. This innovative finding sheds light on the mechanism underlying the effect of meniscus cell lysate on synovial inflammation and OA.

Methods
Cell culture. Human synovial tissues and meniscus samples were obtained from ten patients (eight females with a mean age of 70 years (standard deviation (SD) 6.64), and two males with a mean age of 68 years (SD 7.07)) who suffered from OA of the knee and underwent total knee arthroplasty (TKA). Normal synovial tissue was obtained from the synovial plica of four patients (four males with a mean age of 20 years (SD 4.80)) undergoing arthroscopic surgery to repair a torn anterior cruciate ligament (ACL).

The synovial membranes were minced and incubated with 2 mg/ml type I collagenase on a horizontal shaker at 37°C for eight to ten hours. The cell suspension was seeded and grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 ug/ml streptomycin at 37°C in a 5% CO_2 incubator. At passage 0 (P0), the cells were considered synovial cells. P3 cells were primarily FLSs, and P3 to P5 FLSs were used for the assays.

The menisci were cut into slices and digested with 2 mg/ml collagenase P for eight to 12 hours. The cultivation method was the same as above.

Synovial fluid was aseptically drawn from the knee without any lavage at the beginning of the arthroscopic and TKA surgery. Synovial samples were first collected in tubes containing ethylenediaminetetraacetic acid (EDTA) and immediately centrifuged at 3,000 g to remove cellular debris, and the supernatant was stored at -80°C until assayed.[19] The levels of type I collagen were measured in the synovial fluid using a specific sandwich

enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Shanghai Sangon Industry, China).

Meniscus cell lysate preparation. To lyse cells, the human osteoarthritic meniscus cell suspension was transferred into a centrifuge tube, and was gently rocked on either a rocker or an orbital shaker in the cold room for 15 minutes. After centrifuging the lysate at 12,000 g in a precooled centrifuge for 15 minutes, the supernatant was immediately transferred to a fresh centrifuge tube, and the pellet was discarded. Then, 2×10^5 cells/ml lysed meniscus-treated intact synovial cells were used.[8]

Flow cytometry. For the analysis of apoptosis, approximately 1×10^5 FLSs were resuspended in binding buffer, which included 2 μl of 50 μg/ml propidium iodide (PI) and 2 μl of 20 μg/ml Annexin V-FITC. Following one hour of cell staining, flow cytometric acquisition was performed using a flow cytometer (Beckman Coulter, USA). At least 10,000 cells were collected for each sample at a flow rate of 250 to 300 cells/s, and 488 nm laser excitation was used. Cells were considered apoptotic if they were negative for PI and positive for Annexin V-FITC. The analyses were performed using CytExpert software (Beckman Coulter).

Transfection. OA-FLSs were transfected with siANT3 (50 nM) and positive control sets of small interfering RNA-siANT3 (siNC) (RiboBio, China) as a negative control. Lipofectamine 3000 Transfection Reagent (Invitrogen, Thermo Fisher Scientific, USA) was used to transfect the OA-FLSs according to the manufacturer's instructions. The cells were collected after 48 hours for quantitative real-time polymerase chain reaction (qRT-PCR) analysis and after 72 hours for Western blot (WB) analysis. The sequence was as follows: siANT3_003: ACACGCAGTTCTGGAGGTA. siNC from RiboBio was used as the negative control. All of the reagents were purchased from RiboBio.

ELISA. FLSs were divided into six groups: OA-FLS, OA-FLS + MCL, siNC, siNC+ MCL, siANT3, and siANT3+ MCL. Interleukin (IL)-1β levels in the FLSs were measured using a Human IL-1β Valukine ELISA Kit (Novus, #VAL101) according to the manufacturer's instructions.

RNA extraction, reverse transcription, and qRT-PCR. Total RNA extraction was performed using the miRNeasy Mini Kit (QIAGEN, USA), and complementary DNA (cDNA) was synthesized using the PrimeScript miRNA cDNA Synthesis Kit (Takara Bio, Japan). qRT-PCR was performed using SYBR Premix Ex Taq II (Takara Bio) and a CFX96 qRT-PCR machine. The primers used for analysis are listed in Supplementary Table i. Relative gene expression was calculated using the $2^{-\Delta\Delta t}$ method. All experiments were performed in triplicate.

Western blot analysis. Western blot analysis was carried out as described previously.[20] Total protein was extracted from FLSs using radioimmunoprecipitation assay buffer (RIPA) lysis buffer containing protease and phosphatase inhibitors for 30 minutes. Then, the samples were quantified using a BCA Protein Assay kit (Thermo Fisher Scientific)

and denatured at 100°C for ten minutes. Briefly, 20 μg of protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (MilliporeSigma, USA). The membranes were incubated overnight at 4°C with primary antibodies against ANT3 (1:1,000 dilution; Abcepta, China), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1,000 dilution; Proteintech, USA), ERK (1:2,000 dilution; Cell Signaling Technology (CST), USA), p-ERK (1:1,000 dilution; CST), and IL-1β (1:1,000 dilution; CST). The membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3,000 dilution, CST) at room temperature for one hour.

TUNEL staining. For the apoptosis assay, 1×10^6 cells/well were seeded in six-well plates and cultured at 37°C and 5% CO_2 in a humidified environment. The One Step TUNEL Apoptosis Assay Kit (Meilunbio, China) was used to detect apoptotic cells. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Fluorescence images were acquired with a fluorescence microscope (DMi8; Leica Microsystems, Germany).

Cell growth assay. For cell growth assays, the DNA synthesis rate was assayed by using an EdU Cell Proliferation Kit with Alexa Fluor 488 (Meilunbio) following the manufacturer's instructions. Images were taken and analyzed with a microscope (DMi8; Leica) at ×100 magnification. The ratio of EdU-stained cells (green fluorescence) to Hoechst-stained cells (blue fluorescence) was used to evaluate cell proliferation. For the colony formation assay, 1,000 cells/well were plated in six-well plates and routinely cultured for 14 days. The cells were subsequently fixed with 30% formaldehyde for 15 minutes and stained with 0.1% crystal violet. The number of colonies was determined under an optical microscope.

Immunofluorescence. Immunofluorescence (IF) analysis of the cells was performed as described previously.[21] The antibodies mentioned above were used as primary antibodies, and the secondary antibodies were conjugated to goat anti-rabbit immunoglobulin G (IgG) (Alexa Fluor 555, #4413 S; CST). Images were captured with a confocal laser microscope (LSM 780; ZEISS, Germany) with a 63× magnification.

Destabilization of the medial meniscus-induced OA model. Male wild-type (WT) C57 BL/6 J mice were housed under specific pathogen-free conditions and used in experiments at eight weeks of age. The mice were provided a normal diet and given ad libitum access to water. The mice were anaesthetized by isoflurane inhalation before the operation. Anaesthesia induction was achieved with 2% to 3% isoflurane, and then 1.5% to 2% isoflurane was used for anaesthesia maintenance. Experimental knee osteoarthritis (KOA) was induced in mice by transection of the medial anterior meniscotibial ligament in the left knee as previously described, and sham surgery was used as a control.[22] To exclude the osteoprotective effect of oestrogen in female mice, male mice were selected for all experiments. Briefly, ten male mice were randomly divided into
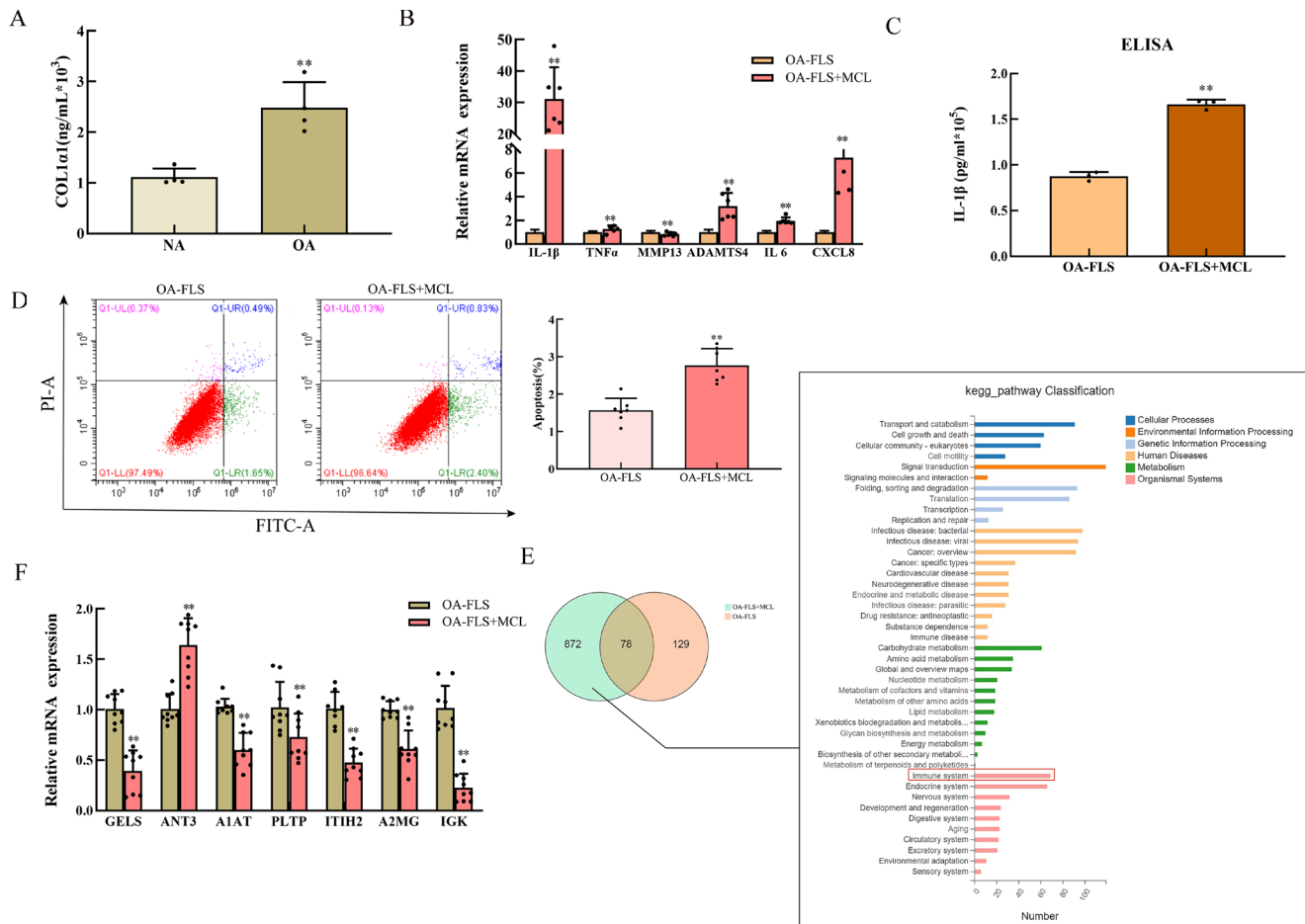
Fig. 1

Meniscal cell lysate (MCL) regulates cellular homeostasis by elevating the level of inflammatory factors in fibroblast-like synoviocytes (FLSs). a) Collagen type I α 1 (COL1a1) protein level evaluation in synovial fluids from normal (NA) and osteoarthritis (OA) patients by enzyme-linked immunosorbent assay (ELISA). b) OA-FLS was incubated with MCL, and the inflammatory factor expressions were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) assay. c) Interleukin (IL)-1β protein level evaluation on MCL-stimulated OA-FLS by ELISA. d) Apoptosis rates were evaluated by flow cytometry after MCL stimulated OA-FLS. e) Synovial cells from three patients as well as proteins isolated from meniscus cells after MCL stimulation of synovial cells were collected and identified by proteomics. f) Quantitative real-time polymerase chain reaction (qRT-PCR) confirmation of seven highly expressed genes in OA + MCL and OA-FLS. Samples from three OA synovial fibroblasts and meniscal each were repeated three times. **$p < 0.01$; p-values were calculated using two-tailed, independent-samples t-test. ADAMTS4, a disintegrin and metalloproteinase with thrombospondin motifs 4; FITC-A, Annexin V-FITC staining; MMP13, matrix metallopeptidase 13; mRNA, messenger RNA; PI-A, propidium iodide staining; TNF, tumour necrosis factor.

two groups (n = 5/cohort): the sham group and the destabilization of the medial meniscus (DMM) group (n = 5/cohort). After eight weeks (mean weight of 17 g (SD 0.3)) the mice were killed, and the knee joints were harvested from these mice and further analyzed by Safranin O and Fast Green staining and immunohistochemical analyses. We have adhered to the ARRIVE guidelines and supplied an ARRIVE checklist to show this (Supplementary Material).

Component analysis of the electrophoretic band by using LC–MS/MS. Liquid chromatography–mass spectrometry (LC–MS)/MS was performed by BGI-Tech (China). Proteins were digested using trypsin (Pierce Biotechnology, USA) at a ratio of 1:50 (mass percentage concentration (w/w), trypsin/protein) for 20 hours at 37°C. Nano-LC–MS/MS experiments were performed on a high-performance liquid chromatography (HPLC) system composed of two

LC-20AD nanoflow liquid chromatography (LC) pumps, an aSIL-20 AC autosampler, and an LC-20AB microflow LC pump (all Shimadzu, Japan) connected to a Q-Exactive HF X mass spectrometer (Thermo Fisher Scientific). The mass spectrometry data were matched with data simulated by the Swiss-Prot/UniProt database with Mascot 2.3.02 to obtain protein results. Gene ontology (GO), clusters of orthologous groups of proteins (COG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) data were also retrieved and analyzed.[23]

Histological analysis and immunohistochemistry. The joints collected for histology were fixed in 4% paraformaldehyde, decalcified in EDTA solution (0.25 mol/l, pH 7.4), and embedded in paraffin. Haematoxylin and eosin (H&E) and safranin-O/fast green staining were conducted with 6 µm-thick tissue sections. Immunohistochemistry was performed following a standard protocol. Briefly, tissue
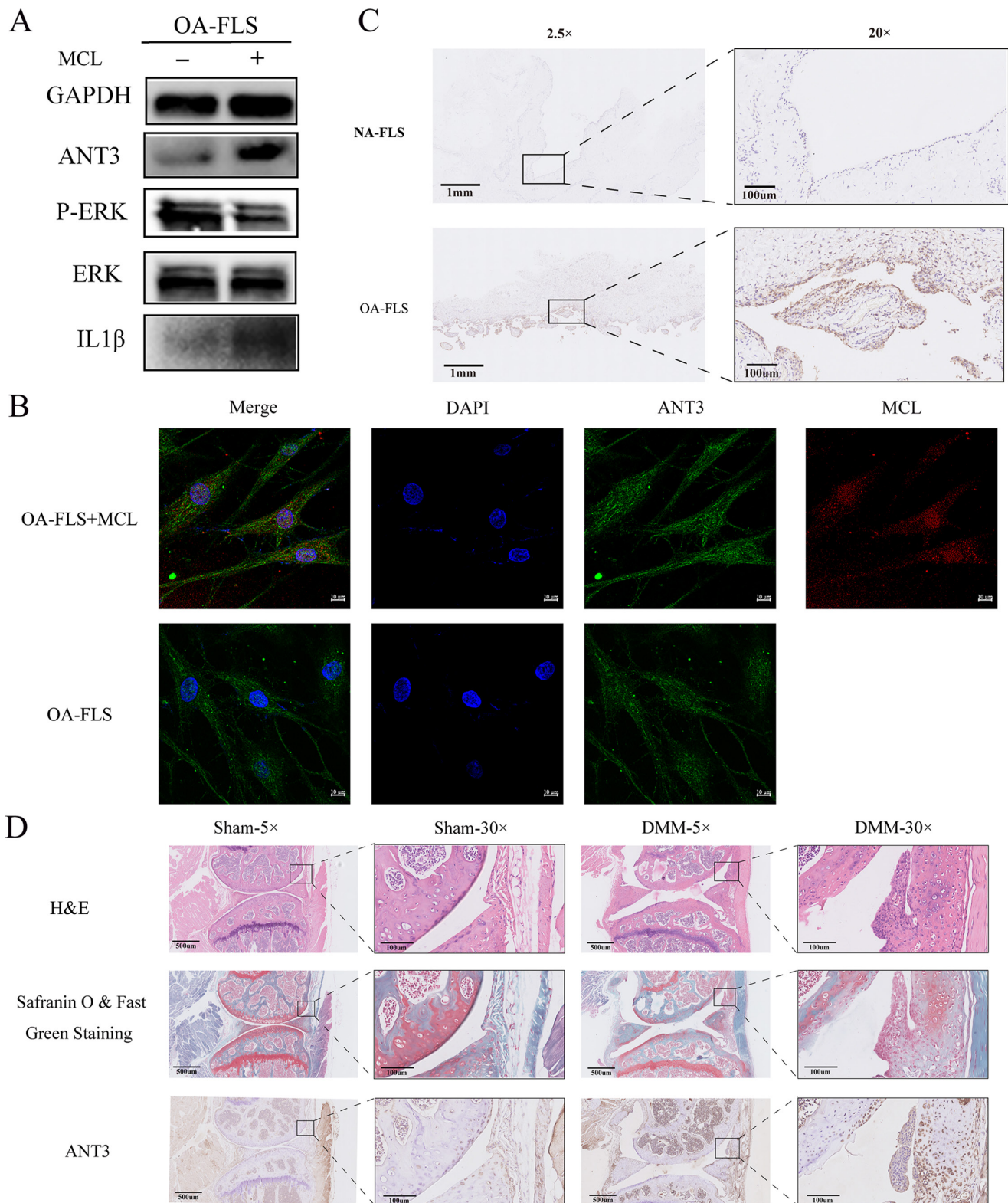
Fig. 2

Fibroblast-like synoviocyte (FLS) adenine nucleotide translocase 3 (ANT3) is upregulated upon meniscal cell lysate (MCL) stimulation in vivo and in vitro. a) Osteoarthritis (OA)-FLSs were pretreated with MCL for 72 hours. The extent of ERK phosphorylation was quantified by Western blotting. b) Immunofluorescence detection of MCL into OA-FLS. c) Representive images of immunohistochemistry (IHC) staining of ANT3 between normal (NA)-FLS and OA-FLS. d) Representative haematoxylin and eosin (H&E) staining, Safranin O, and Fast Green staining, as well as IHC analysis of ANT3 shown in knee joints from mice. DAPI, 4′,6-diamidino-2-phenylindole; DMM, destabilization of the medial meniscus; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin.
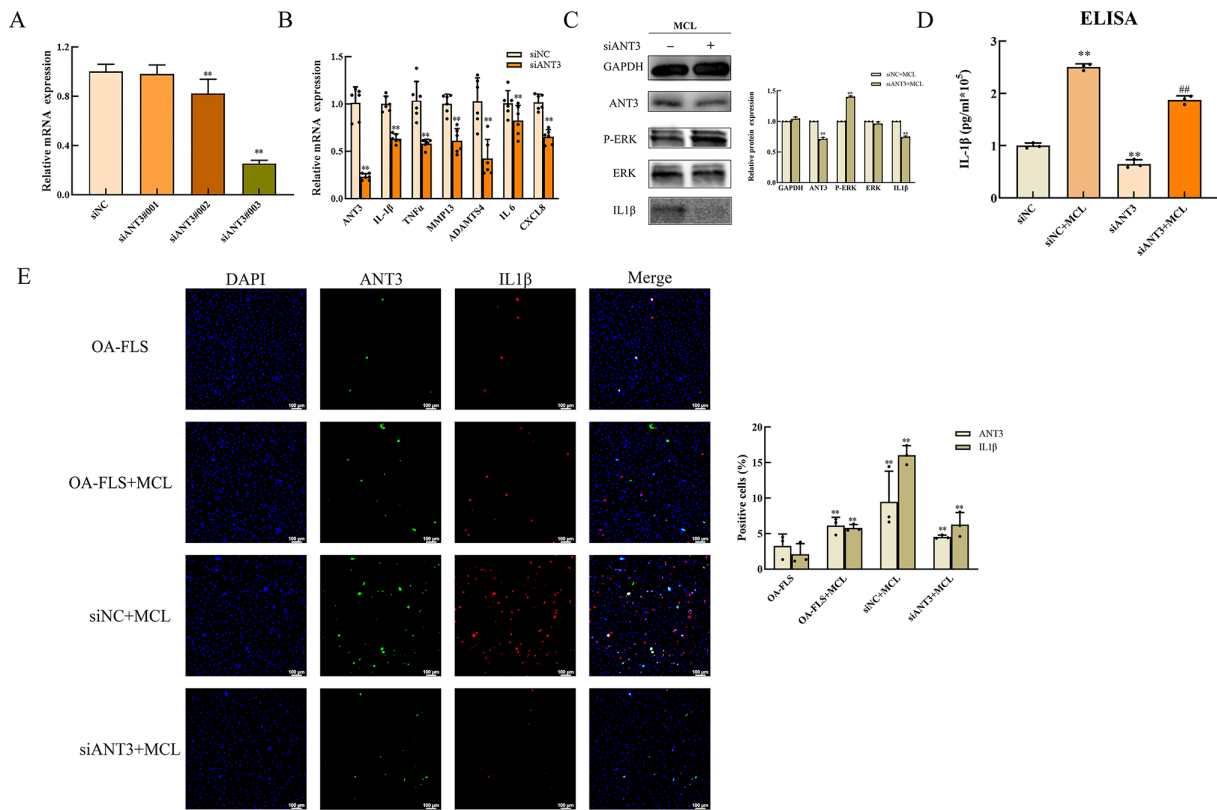
Fig. 3

Adenine nucleotide translocase 3 (ANT3) knockdown alleviated inflammation in fibroblast-like synoviocytes (FLSs) triggered by meniscal cell lysate (MCL). a) Three small interfering RNAs (siRNAs) knockdown efficiency of ANT3 by qRT-PCR. b) qRT-PCR detection of inflammatory factors after knockdown of ANT3. c) Western blot of ERK phosphorylation after knockdown of ANT3. d) Evaluation of interleukin (IL)-1β protein levels after ANT3 knockdown by enzyme-linked immunosorbent assay (ELISA) (**compared with siNC, ##compared with siANT3). e) Representive image of Immunofluorescence analysis of IL-1β after ANT3 knockdown. **p < 0.01, ## p < 0.01; p-values calculated using independent-samples t-test. ADAMTS4, a disintegrin and metalloproteinase withthrombospondin motifs 4; DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP13, matrix metallopeptidase13; mRNA, messenger RNA; siNC, positive control sets of small interfering RNA-siANT3; TNF, tumour necrosis factor.

sections were regularly deparaffinized and rehydrated. The antigen was retrieved with citric acid buffer (pH 6.0) and microwave antigen retrieval for 15 minutes. After blocking with hydrogen peroxide and serum, the tissue sections were incubated with ANT3 antibodies (Abcepta) overnight at 4°C. HRP-labelled secondary antibody was incubated at 37°C for 60 minutes. The colour was developed by applying 3,3′-diaminobenzidine tetrahydrochloride (DAB) (DakoCytomation, USA), and the sections were counterstained with haematoxylin.[24]

Statistical analysis. All experiments were performed with three biological replicates. The data are expressed as the mean (SD). Two-tailed, independent-samples t-tests and Mann-Whitney U tests were used to identify differences between cohorts. One-way analysis of variance (ANOVA) and the Kruskal-Wallis test were carried out for comparisons among multiple groups. Data analysis was performed using SPSS version 20 (IBM, USA). Statistical significance was set at p < 0.05.

Results

MCL triggered synovial inflammation by elevating the levels of inflammatory factors and influencing the viability of FLSs. To investigate the role of MCL in synovitis and OA, we detected the protein level of collagen type I α 1 (COL1A1), a classic identified marker for meniscus cells, in synovial fluids from normal and OA patients. Surprisingly, we discovered that the protein level of COL1A1 was significantly upregulated in the synovial fluid from OA patients. This finding identified the presence of meniscus cell lysate in the synovial fluid (Figure 1a). Moreover, we isolated MCL from the meniscus and stimulated FLSs with MCL. In FLSs, MCL stimulation significantly elevated the levels of several important inflammatory factors, including IL-1β, tumour necrosis factor (TNF)-α, a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4), IL-6, and C-X-C motif chemokine ligand 8 (CXCL8) (Figure 1b). ELISA also revealed that IL-1β levels were elevated in the FLS supernatant upon MCL stimulation (Figure 1c). Furthermore, FLSs showed accelerated apoptosis upon MCL treatment (Figure 1d). These findings indicate that FLSs may exert proapoptotic and proinflammatory effects upon MCL stimulation.

Since MCL plays an important role in exacerbating synovial inflammation, we aimed to uncover the potential targets and the underlying mechanism of this specific
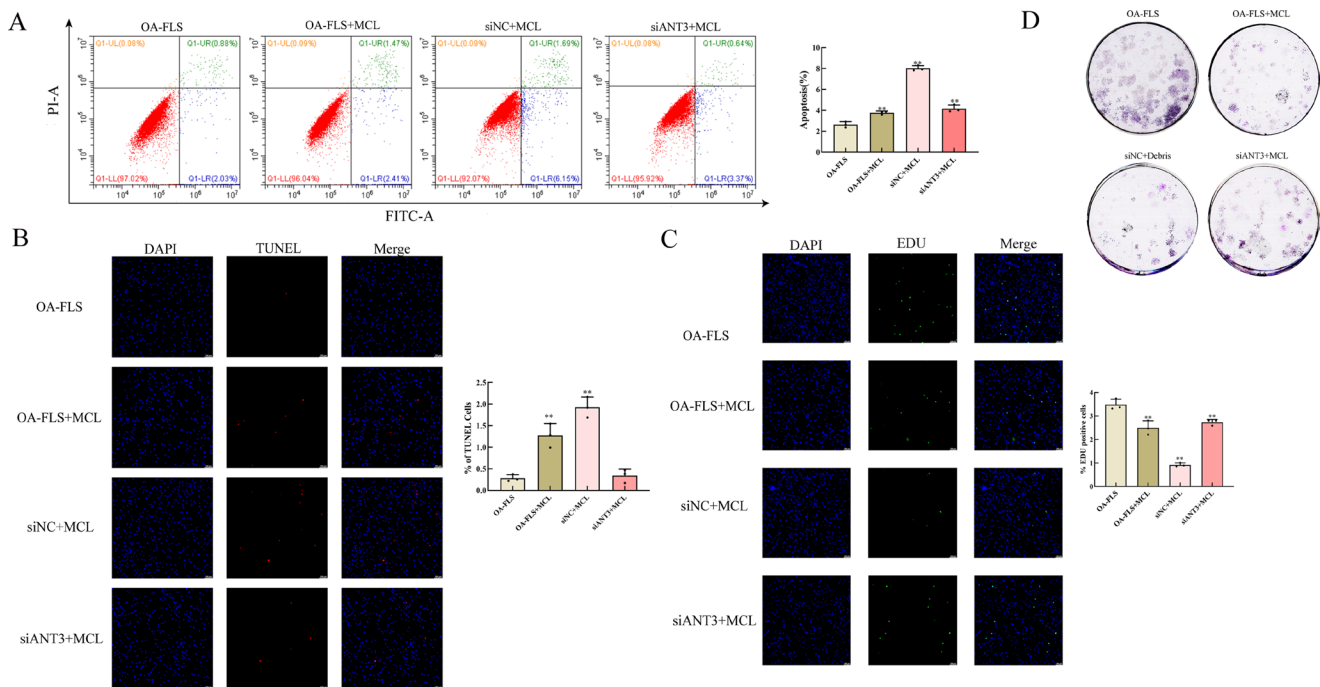
Fig. 4

Silencing adenine nucleotide translocase 3 (ANT3) maintained fibroblast-like synoviocyte (FLS) cellular homeostasis upon meniscal cell lysate (MCL) stimulation. Apoptosis was evaluated by a) flow cytometry and b) terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. Detection of cell proliferation rates was performed using c) EdU assay and d) colony-forming assay (magnification: ×63). e) Western blot analysis of the extent of ERK phosphorylation after hypaphorine treatment, an ERK inhibitor. **p < 0.01; p-values calculated using independent-samples t-test. DAPI, 4',6-diamidino-2-phenylindole; FITC-A, Annexin V-FITC staining; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; siNC, positive control sets of small interfering RNA-siANT3.

phenomenon. (Figure 1e). Through LC–MS, we screened the top seven differentially expressed proteins and found that only the expression of ANT3 was elevated in FLSs during MCL stimulation (Figure 1f).

ANT3 expression in FLSs is upregulated upon MCL stimulation in vitro and is elevated in the synovial tissues of patients and mice with OA. Recently, studies have found that mitochondrial dysfunction is involved in synovitis.[25] ANT3 is located on the mitochondrial inner membrane and exchanges ADP with ATP between the cytosol and the mitochondrial matrix through an electrogenic process, thus the expression of ANT is regulated in response to intrinsic and extrinsic factors regulating cellular metabolism and the patterns of their expression display a tissue-specificity.[20] Consistent with the results shown in Figure 1, WB analysis showed that ANT3 expression was upregulated under MCL treatment (Figure 2a). Confocal imaging of MCL labelled with Cy5 showed that MCL was phagocytized by FLSs, and ANT3 expression was upregulated in FLSs (Figure 2b). In addition, ANT3 expression was significantly upregulated in synovial tissues dissected from OA patients (Figure 2c). These results suggest that ANT3 upregulation in FLSs results from FLS phagocytosis of MCL.

Moreover, we determined ANT3 expression patterns in normal and OA synovial tissues. qRT-PCR, WB, and IF suggested that ANT3 expression was upregulated in

OA-FLSs, and that OA-FLSs exhibited a proinflammatory state in vitro (Supplementary Figure a).

In addition, we validated the ANT3 expression pattern in an animal model. H&E, Safranin O, and Fast Green staining in the mouse DMM model showed aggravated synovitis, and ANT3 was upregulated in the DMM group (Figure 2d).

Silencing ANT3 suppresses the inflammatory response of OA-FLSs. To further validate ANT3 function in FLSs, we used RNA interference and knocked down the expression of ANT3 with three small interfering RNAs (siRNAs). siANT3#003 had the highest knockdown efficiency and was selected for the rest of the experiments (Figure 3a). FLSs were transfected with siANT3, resulting in downregulation of the expression of inflammatory-related genes, as shown by qRT–PCR (Figure 3b). As shown by WB analysis, after ANT3 knockdown the ERK pathway was activated, and IL-1β expression was significantly inhibited (Figure 3c). The level of IL-1β was significantly reduced after ANT3 knockdown, as determined by ELISA (Figure 3d) and IF (Figure 3e). These results suggest that MCL-induced upregulation of ANT3 expression may regulate FLS inflammatory responses.

Knockdown of ANT3 reduces OA-FLS apoptosis and enhances cell proliferation. In addition, we investigated the effects of ANT3 on FLS cellular homeostasis. The apoptosis rate was increased after treatment with MCL
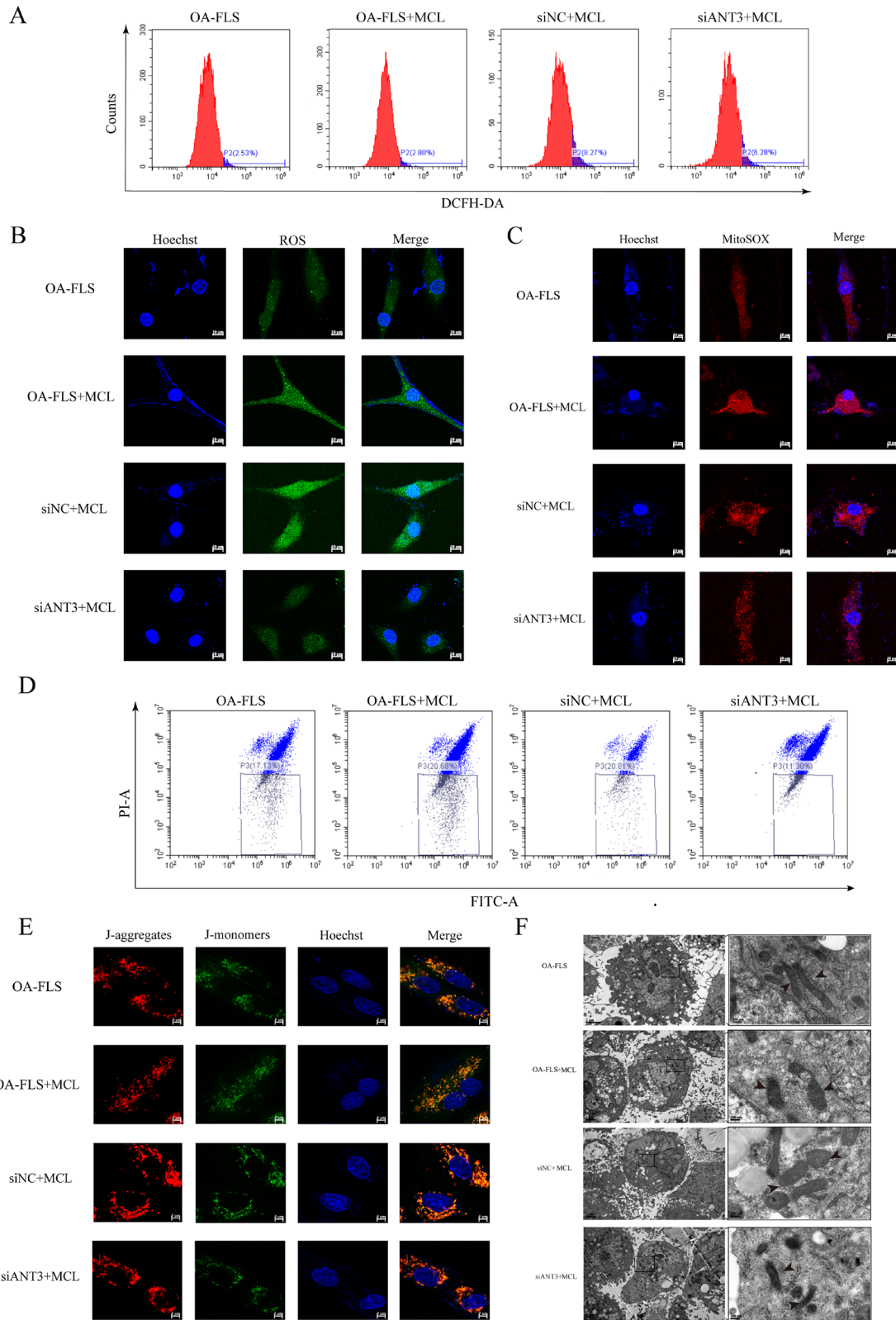
Fig. 5

Knockdown of adenine nucleotide translocase 3 (ANT3) affects mitochondrial function in fibroblast-like synoviocytes (FLSs). The effects of ANT3 on intracellular levels of ROS in osteoarthritis (OA)-FLS by a) flow cytometry and b) immunofluorescence (IF) (magnification: 63×). c) The effect of ANT3 on mitochondrial MitoSOX levels in OA-FLS cells detected by IF (magnification: 63×). The effects of ANT3 on intracellular levels of JC-1 in OA-FLS by d) flow cytometry and e) IF (magnification: 63×). f) Observation of mitochondrial morphological changes after knockdown of ANT3 by transmission electron microscopy (magnification of left- and right-hand images: 3,900× and 37,000×). DCFH-DA, 2'-7'-dichlorodihydrofluorescein diacetate; MCL, meniscal cell lysate.

and significantly decreased after knockdown of ANT3 (Figures 4a and 4b). FLS proliferation rates were significantly decreased after the addition of debris, as determined by the EdU assay (Figures 4c and 4d), while knocking down ANT3 rescued FLS proliferation activity. After addition of the ERK pathway inhibitor hypaphorine (MCE, HY-N2179), knocking down ANT3 could not activate the ERK pathway (Figure 4e).

Aberrant mitochondrial permeability was restored after ANT3 knockdown. Recently, studies have found that mitochondrial dysfunction is involved in synovitis, and cellular homeostasis relies heavily on mitochondria. We further analyzed the effect of ANT3 on mitochondrial function in FLSs. The reactive oxygen species (ROS) level increased significantly after the addition of debris, while ANT3 knockdown alleviated this effect (Figures 5a and 5b). The change in MitoSOX staining was consistent with the change in ROS levels (Figure 5c). We analyzed the mitochondrial membrane potential by staining for JC-1, which is a marker of mitochondrial depolarization in the early apoptotic process, and found that the mitochondrial membrane potential was significantly increased after treatment with MCL and significantly decreased after ANT3 knockdown (Figures 5d and 5e). Transmission electron microscopy showed that mitochondria were swollen after the addition of debris, while the mitochondria were basically unchanged after ANT3 knockdown (Figure 5f).

Discussion

OA is considered a whole-joint disease, and synovitis may activate OA pathogenesis. However, little is known about the crosstalk between the synovium and other joint tissues, such as the meniscus, during OA.[9] In this study, we found a great abundance of MCL in synovial fluid, which could aggravate synovial inflammation in vitro. This finding indicates the pivotal role of MCL in synovitis and the onset of OA. Therefore, elucidating the mechanism of MCL-induced synovitis might provide potential targets for the treatment of early-stage OA.

Synovitis has been reported to occur in most early-stage OA patients and often precedes cartilage degradation and meniscal injury.[21] Once cartilage damage or meniscus injury occurs, cell lysates from cartilage or meniscus are released into synovial fluid and phagocytized by the synovium.[26] FLSs, the primary cells in the synovial membrane, are actively involved in chronic inflammatory responses. During OA, FLSs proliferate rapidly and attach to cartilage and the meniscus. FLSs secrete chemokines, such as IL-1β, TNF-α, and IL-6, to activate other immune cells around the joint to release cartilage-degrading enzymes.[27-29] During OA progression, inflammatory mediators such as IL-1β and inducible nitric oxide synthase (iNOS) increase oxidative stress, and initiate mitochondrial and nuclear DNA damage and epigenetic changes.[28,30] In this study, we were surprised to find that the levels of chemokines, such as IL-1β, TNF-α, and IL-6, in FLSs were significantly elevated after MCL stimulation (synoviocyte inflammatory phenotypes were also regulated by lysates from meniscus debris). Thus, this finding suggests that this articular inflammatory cascade might be triggered by MCL.

For the first time, we identified ANT3 as the most significantly upregulated molecule in MCL-treated FLSs by mass spectrometry. Silencing ANT3 significantly alleviated the inflammatory effect of OA-FLSs. Furthermore, our results show that ANT3 knockdown rescued the apoptosis rate, ROS levels, and mitochondrial membrane potential in FLSs. The ERK pathway has been reported to regulate the expression of ANT3.[20] We demonstrated that MCL blocked the ERK pathway and that knockdown of ANT3 rescued it. Knockdown of ANT3 after the addition of hypaphorine (an inhibitor of ERK) could not be rescued, demonstrating that ANT3 may negatively regulate the ERK pathway. These results suggest that ANT3 plays an important role in regulating synovial inflammation caused by MCL, indicating a new potential target for treating synovitis.

Mitochondrial dysfunction during ageing has been confirmed in chondrocytes and is associated with OA.[28,31,32] An increasing number of studies have shown that ANT3, a member of the ANT family that is located in mitochondrial channels and regulators, acts as a regulator of mitochondria in synoviocytes. During OA, ANT3 levels are elevated in synovial cells. ANT3 silencing modulates mitochondrial recovery, thereby restoring cell viability and alleviating inflammation. However, there are relatively few studies on ANT3 regulation in OA synovitis. This study showed that ANT3 expression was significantly upregulated in OA synovial tissue. ANT3 knockdown significantly mitigated MCL-induced inflammation and significantly decreased the high levels of ROS in MCL-induced synovial inflammation.

However, there are several limitations that need to be addressed. First, because it is difficult to obtain normal synovial tissue from elderly individuals, we chose specimens obtained from young arthroscopic patients (mostly males and those suffering from sports injuries) as the control group (NA), which has certain limitations in the study of mitochondrial dysfunction. Second, more in vivo studies are needed, such as lentivirus and knockout mice that produce ANT3, or whether adding MCL will aggravate OA in mice. In addition, we selected male mice as experimental mice, which has certain limitations. It has been reported that the progress of mouse OA is related to sex; in vivo, ovarian-derived hormones have chondroprotective effects on OA in females, whereas testosterone has a detrimental effect on the severity of OA in males. In the later experiment, we will add female mice to detect the effect of MCL on OA.[33]

More importantly, more in-depth research is required to uncover which cytokines in MCL lead to increased synovitis and the mechanism underlying the ANT3 and ERK pathways. Only then will we be able to treat synovitis with high accuracy and efficiency. Moreover, more OA patients with synovitis need to be enrolled in the database to determine the correlation between ANT3 levels,

synovitis, and the prognosis of OA. Finally, we only discussed the impact of ANT3 in the synovium. Due to the complexity of OA, the overall impact of ANT3 on OA is not clear enough at present. We need to more comprehensively verify the impact of ANT3 on the whole joint microenvironment.

The local low-grade inflammation observed in OA joints has also been considered as a therapeutic target.[2] Epidemiological studies have reported an association between synovial inflammation, OA pain, and structural damage.[34-36] Synovitis can also predict incident cartilage lesions and osteophytes in patients with or without definite OA, and is associated with the risk of joint arthroplasty in patients with symptomatic knee OA.[37] If we can verify that inhibiting ANT3 gene can have a certain effect on OA without affecting other organs in the later stage, ANT3 is a potentially important mediator and drug target in OA.

In summary, MCL was found to increase synovial inflammation and ANT3 expression in FLSs, and ANT3 promoted FLS inflammation in vitro by inhibiting the ERK signalling pathway (Supplementary Figure b). Thus, ANT3-targeted therapies are potential therapeutic approaches for OA via mitigating the deleterious effects of synovitis.

Supplementary material

ⓔ Tables showing primers for quantitative real-time polymerase chain reaction and information regarding patients with total knee arthroplasty and arthroscopic surgery. Figure illustrating that adenine nucleotide translocase 3 was upregulated in osteoarthritis synovial fibroblasts, and graphic illustration summarizing the study's findings.

References

1. **Zong Z**, **Zhang X**, **Yang Z**, et al. Rejuvenated ageing mesenchymal stem cells by stepwise preconditioning ameliorates surgery-induced osteoarthritis in rabbits. *Bone Joint Res*. 2021;10(1):10–21.

2. **Robinson WH**, **Lepus CM**, **Wang Q**, et al. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. *Nat Rev Rheumatol*. 2016;12(10):580–592.

3. **Zhang H**, **Li J**, **Xiang X**, et al. Tert-butylhydroquinone attenuates osteoarthritis by protecting chondrocytes and inhibiting macrophage polarization. *Bone Joint Res*. 2021;10(11):704–713.

4. **Bartok B**, **Firestein GS**. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. *Immunol Rev*. 2010;233(1):233–255.

5. **Wenham CYJ**, **Conaghan PG**. The role of synovitis in osteoarthritis. *Ther Adv Musculoskelet Dis*. 2010;2(6):349–359.

6. **Scanzello CR**, **Goldring SR**. The role of synovitis in osteoarthritis pathogenesis. *Bone*. 2012;51(2):249–257.

7. **Sellam J**, **Berenbaum F**. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nat Rev Rheumatol*. 2010;6(11):625–635.

8. **Wang Q**, **Onuma K**, **Liu C**, et al. Dysregulated integrin $\alpha V \beta 3$ and CD47 signaling promotes joint inflammation, cartilage breakdown, and progression of osteoarthritis. *JCI Insight*. 2019;4(18):18.

9. **Favero M**, **Belluzzi E**, **Trisolino G**, et al. Inflammatory molecules produced by meniscus and synovium in early and end-stage osteoarthritis: a coculture study. *J Cell Physiol*. 2019;234(7):11176–11187.

10. **Clair AJ**, **Kingery MT**, **Anil U**, **Kenny L**, **Kirsch T**, **Strauss EJ**. Alterations in synovial fluid biomarker levels in knees with meniscal injury as compared with asymptomatic contralateral knees. *Am J Sports Med*. 2019;47(4):847–856.

11. **Nair A**, **Gan J**, **Bush-Joseph C**, et al. Synovial chemokine expression and relationship with knee symptoms in patients with meniscal tears. *Osteoarthritis Cartilage*. 2015;23(7):1158–1164.

12. **Kroemer G**, **Galluzzi L**, **Brenner C**. Mitochondrial membrane permeabilization in cell death. *Physiol Rev*. 2007;87(1):99–163.

13. **Leanza L**, **Zoratti M**, **Gulbins E**, **Szabo I**. Mitochondrial ion channels as oncological targets. *Oncogene*. 2014;33(49):5569–5581.

14. **Halestrap AP**, **Brenner C**. The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. *Curr Med Chem*. 2003;10(16):1507–1525.

15. **Stepien G**, **Torroni A**, **Chung AB**, **Hodge JA**, **Wallace DC**. Differential expression of adenine nucleotide translocator isoforms in mammalian tissues and during muscle cell differentiation. *J Biol Chem*. 1992;267(21):14592–14597.

16. **Dolce V**, **Scarcia P**, **Iacopetta D**, **Palmieri F**. A fourth ADP/ATP carrier isoform in man: identification, bacterial expression, functional characterization and tissue distribution. *FEBS Lett*. 2005;579(3):633–637.

17. **Guo X**, **Huang Y**, **Qi Y**, et al. Human cytomegalovirus miR-UL36-5p inhibits apoptosis via downregulation of adenine nucleotide translocator 3 in cultured cells. *Arch Virol*. 2015;160(10):2483–2490.

18. **Yang Z**, **Cheng W**, **Hong L**, et al. Adenine nucleotide (ADP/ATP) translocase 3 participates in the tumor necrosis factor induced apoptosis of MCF-7 cells. *Mol Biol Cell*. 2007;18(11):4681–4689.

19. **Turati M**, **Maggioni D**, **Zanchi N**, et al. Characterization of synovial cytokine patterns in bucket-handle and posterior horn meniscal tears. *Mediators Inflamm*. 2020;2020:5071934.

20. **Wu P-K**, **Hong S-K**, **Chen W**, et al. Mortalin (HSPA9) facilitates BRAF -mutant tumor cell survival by suppressing ANT3-mediated mitochondrial membrane permeability. *Sci Signal*. 2020;13(622):622.

21. **Estell EG**, **Silverstein AM**, **Stefani RM**, et al. Cartilage wear particles induce an inflammatory response similar to cytokines in human fibroblast-like synoviocytes. *J Orthop Res*. 2019;37(9):1979–1987.

22. **Glasson SS**, **Blanchet TJ**, **Morris EA**. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis Cartilage*. 2007;15(9):1061–1069.

23. **Xu R**, **Liang J**, **Luo Y**, et al. Mass spectrometry identification of potential biomarker proteins in the 150-kD electrophoretic band in patients with schizophrenia. *Medicine (Baltimore)*. 2018;97(51):e13553.

24. **Zhang Q**, **Xiang E**, **Rao W**, et al. Intra-articular injection of human umbilical cord mesenchymal stem cells ameliorates monosodium iodoacetate-induced osteoarthritis in rats by inhibiting cartilage degradation and inflammation. *Bone Joint Res*. 2021;10(3):226–236.

25. **Bao J**, **Yan W**, **Xu K**, et al. Oleanolic acid decreases IL-1beta-induced activation of fibroblast-like synoviocytes via the SIRT3-NF-kappab axis in osteoarthritis. *Oxid Med Cell Longev*. 2020;2020:7517219.

26. **Kuster MS**, **Podsiadlo P**, **Stachowiak GW**. Shape of wear particles found in human knee joints and their relationship to osteoarthritis. Rheumatology. 1998;37(9):978–984.

27. **Rahmati M**, **Mobasheri A**, **Mozafari M**. Inflammatory mediators in osteoarthritis: a critical review of the state-of-the-art, current prospects, and future challenges. *Bone*. 2016;85(1):81–90.

28. **Cheleschi S**, **Gallo I**, **Barbarino M**, et al. MicroRNA mediate visfatin and resistin induction of oxidative stress in human osteoarthritic synovial fibroblasts via NF-κB pathway. *Int J Mol Sci*. 2019;20(20):5200.

29. **Liu S**, **Cao C**, **Zhang Y**, et al. Pi3K/Akt inhibitor partly decreases TNF-α-induced activation of fibroblast-like synoviocytes in osteoarthritis. *J Orthop Surg Res*. 2019;14(1):425.

30. **D'Adamo S**, **Cetrullo S**, **Borzì RM**, **Flamigni F**. Effect of oxidative stress and 3-hydroxytyrosol on DNA methylation levels of miR-9 promoters. *J Cell Mol Med*. 2019;23(11):7885–7889.

31. **Castro CM**, **Corciulo C**, **Solesio ME**, **Liang F**, **Pavlov EV**, **Cronstein BN**. Adenosine A2A receptor (A2AR) stimulation enhances mitochondrial metabolism and mitigates reactive oxygen species-mediated mitochondrial injury. *FASEB J*. 2020;34(4):5027–5045.

32. **Fernández-Moreno M**, **Soto-Hermida A**, **Vázquez-Mosquera ME**, et al. Mitochondrial DNA haplogroups influence the risk of incident knee osteoarthritis in OAI and CHECK cohorts. A meta-analysis and functional study. *Ann Rheum Dis*. 2017;76(6):1114–1122.

33. **Ma H-L**, **Blanchet TJ**, **Peluso D**, **Hopkins B**, **Morris EA**, **Glasson SS**. Osteoarthritis severity is sex dependent in a surgical mouse model. *Osteoarthritis Cartilage*. 2007;15(6):695–700.

34. **Hill CL**, **Hunter DJ**, **Niu J**, et al. Synovitis detected on magnetic resonance imaging and its relation to pain and cartilage loss in knee osteoarthritis. *Ann Rheum Dis*. 2007;66(12):1599–1603.

35. **Stoppiello LA**, **Mapp PI**, **Wilson D**, **Hill R**, **Scammell BE**, **Walsh DA**. Structural associations of symptomatic knee osteoarthritis. *Arthritis Rheumatol*. 2014;66(11):3018–3027.

36. **Sarmanova A**, **Hall M**, **Moses J**, **Doherty M**, **Zhang W**. Synovial changes detected by ultrasound in people with knee osteoarthritis - a meta-analysis of observational studies. *Osteoarthritis Cartilage*. 2016;24(8):1376–1383.

37. **Latourte A**, **Kloppenburg M**, **Richette P**. Emerging pharmaceutical therapies for osteoarthritis. *Nat Rev Rheumatol*. 2020;16(12):673–688.

Author information:
- X. Du, MS, Researcher, Department of Joint Surgery, First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China; Department of Parasitology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China; Guangdong Provincial Key Laboratory of Orthopedics and Traumatology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China.
- Z. Jiang, MD, Researcher
- G. Fang, MD, Researcher
- R. Liu, MS, Researcher
- X. Wen, MD, Researcher
- Z. Zhang, MD, PhD, Orthopaedic Surgeon
  Department of Joint Surgery, First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China; Guangdong Provincial Key Laboratory of Orthopedics and Traumatology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China.
- Y. Wu, PhD, Researcher, Department of Parasitology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China.
- S. Hu, PhD, Orthopaedic Surgeon, Department of Joint Surgery, The Third Affiliated Hospital of Southern Medical University, Guangzhou, China.

Author contributions:
- X. Du: Conceptualization, Data curation, Writing – original draft.
- Z. Jiang: Methodology, Formal analysis.
- G. Fang: Software, Visualization.
- R. Liu: Validation, Resources.
- X. Wen: Visualization.
- Y. Wu: Project administration.
- S. Hu: Writing – review & editing.
- Z. Zhang: Supervision, Funding acquisition.
- X. Du and Z. Jiang contributed equally to this work.

ICMJE COI statement:
- The authors declare no conflicts of interest related to this manuscript.

Data sharing:
- Data are contained within the article and Supplementary Material.

Ethical review statement:
- All of the patients signed an informed consent form, and the study was approved by the Ethics Committee of the First Affiliated Hospital of Medical College of Sun Yat-sen University, Guangzhou, China. The animal experiments performed in the present study adhered to protocols approved by the Institutional Review Board of Sun Yat-sen University (SYSU-IACUC-2020-000504); male wild-type (WT) C57 BL/6J mice were used (purchased from GemPharmatech, China).