MAGL regulates synovial macrophage polarization vis inhibition of mitophagy in osteoarthritic pain

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Abstract. Pain is the hallmark symptom of osteoarthritis (OA), and current analgesic treatments may be insufficient or have potentially adverse effects. The inhibition of Monoacylglycerol lipase (MAGL) produces anti-inflammatory and anti-nociceptive effects. However, the potential mechanism of MAGL in OA pain remains unclear. In the present study, the synovial tissues were removed from OA patients and mice. Immunohistochemical staining and western blotting were used to detect the expression of MAGL. M1 and M2 polarization markers were detected by flow cytometry and western blotting, and the mitophagy levels were detected by the immunofluorescence staining of mitochondrial autophagosomes with lysosomes and western blotting. The OA mice were intraperitoneally injected with MJN110 to inhibit MAGL once a day for a week. Mechanical and thermal pain thresholds were detected by electronic Von Frey and hot plate methods on days 0, 3, 7, 10, 14, 17, 21, and 28. The accumulation of MAGL in the synovial tissues of OA patients and mice promoted the polarization of macrophages towards an M1 phenotype. Pharmacological inhibition and siRNA knockdown of MAGL promoted polarization of M1 macrophages towards an M2 phenotype. MAGL inhibition increased the mechanical and thermal pain thresholds of OA mice and enhanced the mitophagy levels of M1 macrophages. In conclusion, in the present study, it was shown that MAGL regulated synovial macrophage polarization by inhibiting mitophagy in OA.

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Abbreviations: OA, osteoarthritis; MAGL, monoacylglycerol lipase; 2-AG, 2-arachidonoylglycerol; MIA, monoiodoacetate; iNOS, inducible nitric oxide synthase; Arg1, arginase 1; H&E, hematoxylin and eosin; PINK1, PTEN-induced kinase 1

Key words: MAGL, macrophage polarization, mitophagy, osteoarthritis, pain

Introduction

Osteoarthritis (OA) is a chronic degenerative joint disease that causes stiffness, restrictions in joint motion, and chronic persistent pain (1,2). Pathologically, OA is characterized by progressive articular cartilage degeneration, subchondral bone rebuilding, and synovial inflammation (3). Worldwide, knee OA is a growing public health issue and a major cause of disability in the elderly (4,5). Chronic pain is a hallmark symptom of OA, which often necessitates the need for medical care and contributes to a reduced quality of life, and increased healthcare costs. However, current analgesic treatments for OA pain are often insufficient or have potentially severe adverse effects. Therefore, there is an essential need to elucidate the mechanisms of OA pain.

Recent studies have shown that synovial inflammation and chondrocyte cell death play prominent roles in OA progression (6-8). Synovial macrophages can polarize towards the proinflammatory M1 phenotype and secrete proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6, further accelerating cartilage degeneration (9). Inhibiting the polarization of synovial inflammation to the proinflammatory M1 phenotype may be a vital strategy to alleviate synovial inflammation and OA pain. Monoacylglycerol lipase (MAGL) is the enzyme responsible for breaking down 2-arachidonoylglycerol (2-AG), and the inhibition of MAGL enhances endocannabinoid signaling, reduces the levels of proinflammatory metabolites, and produces anti-inflammatory and anti-nociceptive effects (10-13). Studies have found that the inhibition of MAGL can reduce acute inflammatory pain and alleviate joint inflammation and pain (14,15). However, the influence and mechanism of MAGL on the polarization of synovial macrophages in OA remain to be further studied.

Mitophagy, or mitochondrial autophagy, is a selective process that mitigates inflammation and maintains homeostasis by delivering damaged mitochondria to autophagosomes

for destruction (16,17). Studies have shown that mitophagy is related to pain relief, including neuropathic pain and low back pain (18-20). In addition, it was found that enhanced mitophagy reversed LPS/IFN-γ-mediated M1 activation of macrophages (21). However, whether MAGL influences the levels of mitophagy of synovial macrophages in OA remains unclear. Thus, here it was hypothesized that MAGL may regulate the polarization of synovial macrophages by targeting mitophagy.

In the present study, the primary aim was to explore the potential role of MAGL in OA pain. MAGL accumulation in synovial tissue and M1 polarization of synovial macrophages was observed in OA patients and monoiodoacetate (MIA) mice model of OA. Next, the effects of pharmacological inhibition and MAGL knockdown on the polarization of macrophages were xassessed. Lastly, it was determined that MAGL regulated the polarization of macrophages by targeting mitophagy. These findings may provide a novel perspective on the mechanism of MAGL for OA pain.

Materials and methods

Human OA patients. Written informed consent was obtained from each patient for inclusion in the study and the use of their synovial tissues for research. Synovial tissues were collected from patients who underwent total knee replacement due to knee OA (OA group, n=5; one woman, four men; age range, 64-74 years; median age, 69 years) and those who underwent knee arthroscopic surgery due to anterior cruciate ligament injury (Control group, n=5; one woman, four men; age range, 63-71 years; median age, 66 years). This study was performed in accordance with the Ethical Standards of the Declaration of Helsinki and was approved by the Ethics Committee of Suzhou Municipal Hospital (approval no. K-2021-066-K01).

Animal model. Male C57BL/6 (C57) mice weighing 25-35 g were obtained from the Sino-British SIPPR/BK Lab (Shanghai, China). All mice were provided *ad libitum* access to food and water, housed at a temperature of 23-25˚C and a humidity of 45-55%, with a 12/12-h light/dark cycle for 1 week prior to experimentation. The mice were randomly divided into three groups (n=5 per group): Control group, an MIA-OA group (MIA), and an MJN110 group (MIA + MJN110).

The MIA model of OA pain in the mouse was established as previously described (22). Briefly, the mice in the MIA-OA and MJN110 groups were anesthetized with 50 mg/kg pentobarbital sodium (intraperitoneal injection). The ipsilateral knee joint was trimmed and wiped with alcohol, the knee was kept in a bent position to identify the precise site for injection, and the 26 G needle was inserted to inject MIA (0.1 mg/20 μl) (MilliporeSigma) through the patellar tendon to the gap beneath the patella. A total of 3 weeks after the MIA injection, the mice in the MJN110 group were intraperitoneally injected with 1 mg/kg MJN110 (MilliporeSigma) once a day for a week. On day 28, the mice were sacrificed (CO_2 euthanasia, the container was gradually filled with carbon dioxide at a rate of 49.5% vol/min) for synovial tissue collection.

All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (approval no. Y20210267).

Polarization of macrophages. In the logarithmic growth phase, macrophages (RAW264.7, Beyotime Institute of Biotechnology, cat. no. C7505) were selected and seeded into 6-well plates. After cell adhesion, the cell culture medium (DMEM/F12, 10% FBS, 1% Penicillin-Streptomycin) (Gibco; Thermo Fisher Scientific, Inc.) was discarded and washed once with PBS. Then, the M1 polarization induction medium containing 40 ng/ml LPS (MilliporeSigma) or M2 polarization induction medium containing 40 ng/ml IL-4 (MedChemExpress) was added as appropriate (23-25). Finally, the macrophages were incubated with 5% CO_2 at 37˚C for 36 h.

siRNA-mediated knockdown of MAGL in macrophages. MAGL-siRNA was designed and synthesized by Gima Gene Company and divided into a Control group (untransfected), a siRNA Negative Control group (NC siRNA, sense 5'-UUC UCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGA CACGUUCGGAGAATT-3'), and three target gene transfection groups (MAGL siRNA 1 sense, 5'-GCUGGACAUGCU GGUAUUUTT-3' and antisense, 5'-AAAUACCAGCAUGUC CAGCTT-3'; MAGL siRNA 2, sense 5'-CCAUGACCAUGU UGGCCAUTT-3' and antisense, 5'-AUGGCCAACAUGGUC AUGGTT-3'; and MAGL siRNA 3 sense, 5'-GCCUACCUG CUCAUGGAAUTT-3' antisense, 5'-AUUCCAUGAGCAGGU AGGCTT-3'). Macrophages in each group were seeded in 6-well plates. The following experiments were performed in accordance with the instructions of the Gima gene product. The siRNA was diluted with buffer solution and gently mixed to prepare the siRNA transfection diluent. Opti-MEM (200 μl) was diluted with Lipofectamine® 3000 (5 μl) (Invitrogen; Thermo Fisher Scientific, Inc.) and mixed and incubated for 5 min at room temperature. The diluted Lipofectamine® with 100 pmol siRNA was gently mixed and incubated for 20 min. The mixed solution of the complex was added to the cell culture plate, and the cells were incubated for 48 h.

Flow cytometry. The cells were incubated in blocking buffer (0.5% BSA in 1x PBS) for 30 min at room temperature and stained with FITC-conjugated anti-inducible nitric oxide synthase (iNOS; 1:20, BD Biosciences, cat. no. 610330) and PE-conjugated anti-arginase 1 (Arg1; 1:20, R&D Systems, cat. no. IC5868P) at 4˚C in the dark for 30 min. The cells were analyzed using a BD FACSAria™ II flow cytometer (BD Biosciences) and the BD FACSDiva™ software version 8.0 (BD Biosciences).

Immunohistochemical staining. Immunohistochemistry was performed as previously described (26). Briefly, the synovial tissues were cut into 7 μm thick sections. After blocking in 5% goat serum in PBS for 1 h at room temperature, the tissue sections were incubated with primary antibodies against anti-MAGL (1:100, Abcam, cat. no. ab246902), anti-iNOS (1:200, ProteinTech Group, Inc., cat. no. 22226-1), anti-Arg1 (1:200, ProteinTech Group, Inc., cat. no. 16001-1), anti-CD80 (1:300, ProteinTech Group, Inc., cat. no. 14292-1), or anti-CD206 (1:300, ProteinTech Group, Inc., cat. no. 18704-1) overnight at 4˚C followed by the secondary antibody (1:1,000, Abcam, cat. no. ab6721) for 2 h at room temperature. An Olympus CH30 (Olympus Corporation) microscope was used to capture images (x200 or x400).

Hematoxylin and eosin (H&E) staining. H&E staining was performed and evaluated as previously described (27). The synovial tissues of the OA patients or OA mice were fixed with 4% paraformaldehyde for 24 h at 4˚C and decalcified in 10% EDTA for 2 weeks. The synovial tissues were embedded in paraffin before cutting into 7 μm thick sections and then stained with H&E. Evaluations were performed using an Olympus CH30 microscope (x200 or x400). The inflammation score was evaluated by scoring the severity of the infiltration of inflammatory cells as follows: 0, no; 1, mild; 2, moderate; 3, severe.

Western blotting. Western blotting was performed as previously described (28). The protein concentrations of the synovial tissues or cells were determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Proteins (30 μg) were separated by SDS-PAGE on a 10% gel and transferred to PVDF membranes (MilliporeSigma). The PVDF membranes were blocked with 5% non-fat dry milk for 2 h, followed by incubation with one of the following primary antibodies at 4˚C overnight: Anti-MAGL (1:1,000, Abcam, cat. no. ab246902), anti-iNOS (1:1,000, ProteinTech Group, Inc., cat. no. 22226-1), anti-Arg1 (1:5,000, ProteinTech Group, Inc., cat. no. 16001-1), anti-TNF-α (1:1,000, ABclonal, cat. no. A20851), anti-IL-1β (1:1,000, ABclonal, cat. no. A16288), anti-IL-6 (1:1,000, ABclonal, cat. no. A11115), anti-PTEN-induced kinase 1 (PINK1) (1:1,000, ProteinTech Group, Inc., cat. no. 23274-1), anti-Parkin (1:1,000, ProteinTech Group, Inc., cat. no. 14060-1), or anti-β-actin (1:5,000, ProteinTech Group, Inc., cat. no. 81115-1). The following day, the membranes were washed and incubated with the secondary antibody (1:5,000, Abcam, cat. no. ab205718) for 2 h at room temperature. The proteins were detected using Pierce ECL Western Blotting Substrate (Thermo Fisher).

Immunofluorescence staining. The synovial tissues were permeabilized in 0.1% Triton X-100 for 20 min and then blocked with 5% bovine serum albumin for 1 h at room temperature. Then the synovial tissues were incubated with anti-MAGL (1:200, Abcam, cat. no. ab246902) and anti-iNOS (1:200; Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. MA5-17139) antibodies overnight at 4˚C, followed by incubation with the secondary antibody (1:1,000, Abcam, cat. nos. ab150084 and ab150113) for 2 h at room temperature in the dark. An Olympus Fluoview FV3000 (Olympus Corporation) confocal microscope was used to capture images.

Transmission electron microscopy. The synovial tissues were cut into 1 mm^3 segments, preserved in 3% glutaraldehyde for 2 h at 4˚C, treated with 1% osmic acid for 2 h at 4˚C, and dried with various acetone concentrations before encasing in resin. The samples were sliced into extremely thin sections (90 nm) using an ultramicrotome. A Hitachi H-7560 (Hitachi) transmission electron microscope was used to capture images.

Mechanical threshold test. The mechanical pain threshold of mice was measured using an electronic von Frey (Ugo Basile S.R.L, cat. no. 38450) (29). Briefly, the mice were individually placed in a cage with a grid floor for 1 h to adapt to the new environment. An increasing force (g) was applied on the plantar surface of the hind using rigid 0.5 mm diameter polypropylene tips. The mechanical threshold was based on the pressure at which paw withdrawal occurred. This test was done before, and on days 3, 7, 10, 14, 17, 21, and 28 after the injection of MIA. The mechanical threshold was tested thrice, and the mean value was used each time.

Thermal threshold test. The thermal pain threshold of mice was measured using a hot plate (Ugo Basile S.R.L., cat. no. 35250) as described previously (30). The mice were placed on a metal surface of the hot plate equipment maintained at 55±0.1˚C. The hot plate was surrounded by a transparent plastic barrier. The latency to jumping off the plate or licking a hind paw was recorded; 30 seconds was used as a cut-off time to protect the paws against injury.

Statistical analysis. All experiments were performed at least three times. Data are presented as the mean ± SD. Statistical analysis was performed using GraphPad Prism version 8.0.1 (GraphPad Software, Inc.). Normality and homogeneity were evaluated using Shapiro-Wilk and Levene tests, respectively. Differences between groups were compared using an unpaired Student's t-test (two groups), a Mann-Whitney U test (two groups), a Kruskal-Wallis followed by Dunn's test, or a one-way or two-way ANOVA followed by a Tukey's post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MAGL accumulates in patients with knee OA with the polarization of macrophages towards an M1 phenotype. To investigate the potential role of MAGL in OA, we first collected synovial tissues from patients who underwent total knee replacement due to knee OA (OA group, n=5) and from patients who underwent arthroscopic knee surgery due to anterior cruciate ligament injury (Control group, n=5) to perform H&E staining. As shown in Fig. 1A and B, the synovial tissues of the OA group were thickened, and there was a greater degree of infiltration of inflammatory cells compared with the Control group. Additionally, the synovial tissue score in the OA group was significantly increased compared with that of the Control group ($P<0.01$). Immunohistochemical staining and western blotting were performed to identify the MAGL levels in the synovial tissue. As shown in Fig. 1C and D, compared with the Control group, the average optical density of MAGL in the OA group was significantly increased ($P<0.01$). The protein expression levels of MAGL in the synovial tissues of the Control group were low, while that of the OA group was significantly higher ($P<0.01$) (Fig. 1E and F).

To further determine the polarization phenotype of synovial macrophages from knee OA patients, the expression levels of the M1 polarization markers, iNOS and CD80, and the M2 polarization markers, Arg1 and CD206, were determined by immunohistochemical staining. As shown in Fig. 1G and H, compared with the Control group, the mean optical density of iNOS and CD80 in the OA group was significantly higher ($P<0.01$). The expression levels of Arg1 and CD206 were low in the synovial tissues of both the Control group and the OA group, and there was no significant difference in the average

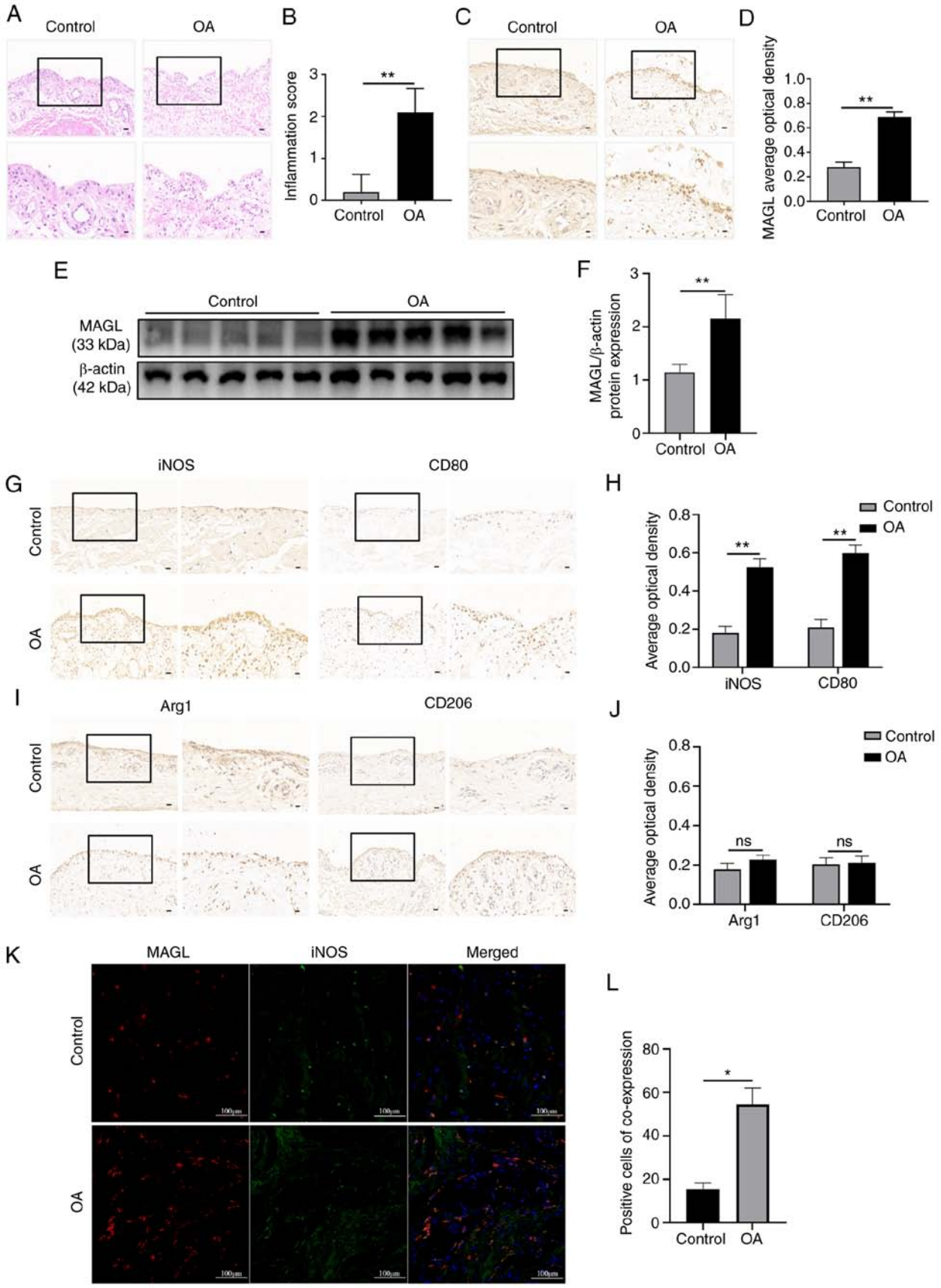

Figure 1. MAGL accumulates in OA patients and polarizes macrophages towards an M1 phenotype. (A) Representative images of the synovial tissues stained with H&E (upper panel, x200, scale bar 50 μm; lower panel, x400, scale bar 20 μm). (B) Inflammation scores of the synovial tissues. (C and D) Immunohistochemical staining for MAGL in synovial tissues (upper panel, x200, scale bar 50 μm; lower panel, x400, scale bar 20 μm). (E) Western blots of MAGL expression in synovial tissues. (F) Western blotting analysis of MAGL in synovial tissues of each group. (G-J) Immunohistochemical staining of iNOS, CD80, Arg1, and CD206 in synovial tissues (left x200, scale bar 50 μm; right x400, scale bar 20 μm). (K and L) Immunofluorescence staining of MAGL (red) with iNOS (green) in the synovial tissues (scale bar 100 μm). n=5 per group. *P<0.05, **P<0.01 vs. control group. ns, not significant. MAGL, monoacylglycerol lipase; OA, osteoarthritis.

optical density between the Control group and OA group (P>0.05) (Fig. 1I and J). Furthermore, it was found that the colocalization of MAGL with the M1 macrophage marker iNOS in the synovial tissues of the OA group increased when compared with that of the Control group (P<0.05) (Fig. 1K and L). Hence, these results indicated that MAGL was upregulated in patients with knee OA, with synovial macrophages exhibiting polarization towards an M1 phenotype.

Pharmacological inhibition of MAGL promotes polarization of macrophages towards an M2 phenotype and alleviates pain behaviors in OA mice. To further investigate the role of MAGL in OA, MJN110, a potent pharmacological inhibitor of MAGL, was intraperitoneally injected in MIA-induced OA mice once a day for a week. First, the expression levels of MAGL in the synovial tissue were detected. As shown in Fig. 2A and B, compared with the Control group, the average optical density of MAGL in the MIA-OA group was significantly increased (P<0.01), and the average optical density of MAGL in the MJN110 group was statistically significantly lower than that in MIA-OA group (P<0.01). Next, the effect of MAGL inhibition on the polarization of synovial macrophages in MIA-induced OA mice was investigated by detecting the expression levels of iNOS and Arg1 in the synovial tissues. As shown in Fig. 2C and D, the mean optical density of iNOS in the MIA-OA group was significantly increased compared with the Control and MJN110 groups (P<0.01). There was no significant difference in the expression levels of Arg1 in the Control group and the MIA-OA group (P>0.05). Compared with the MIA-OA group, the average optical density of Arg1 in the MJN110 group was significantly increased (P<0.01) (Fig. 2E and F).

To further identify the role of MAGL inhibition in OA pain treatment, H&E staining, and mechanical and thermal threshold tests were performed. Based on the staining results (Fig. 2G and H), it was found that the synovial tissue score of the MJN110 group was significantly lower when compared with the MIA-OA group (P<0.01). The results of the mechanical pain and thermal pain tests are shown in Fig. 2I and J. Compared with the Control group, the thresholds of mechanical and thermal pain in the MIA-OA group were significantly reduced after 7 days of MIA injection, which lasted until day 28 (P<0.05). After 7 days of continuous administration of MJN110, the thresholds of mechanical and thermal pain were significantly higher than those of the MIA-OA group (P<0.05). These results suggest that the inhibition of MAGL promotes the polarization of synovial macrophages towards an M2 phenotype and alleviates pain behaviors in OA mice.

MAGL accumulates in M1-polarized mice macrophages in vitro. Next, the function of MAGL *in vitro* was explored. Mice macrophages were divided into a Control group, the M1 group (LPS polarization inducing), and the M2 (IL-4 polarization inducing) group. The representative pictures of the cellular morphologies of the three groups are shown in Fig. 3A. Initially, flow cytometry experiments were used to verify the effectiveness of the induction medium. As shown in Fig. 3B, compared with the M2 group, the proportion of iNOS-positive cells in the M1 group was significantly higher. In comparison, the proportion of Arg1-positive cells in the M1 group was significantly lower than that in the M2 group (P<0.01). Meanwhile, western blotting was used to determine the trends of protein levels of iNOS and Arg1 in macrophages in the three groups (Fig. 3C-E).

Subsequently, western blotting was used to examine the expression of MAGL in the macrophages in the three groups. As shown in Fig. 3F-G, compared with the Control group, the protein expression levels of MAGL in the macrophages of the M1 group significantly increased. In contrast, the protein expression levels of MAGL of the M2 group significantly decreased compared with that of the M1 group (P<0.01).

MAGL knockdown suppresses the polarization of M1 macrophages and promotes polarization towards an M2 phenotype in vitro. Mice macrophages (LPS polarization inducing) were transfected with MAGL siRNA or NC siRNA (Fig. 4A). Western blotting and flow cytometry experiments confirmed the effect on the polarization of macrophages. As shown in Fig. 4B-E, the protein expression levels of MAGL and iNOS in the LPS group significantly increased compared with the Control group (P<0.01), and the protein expression levels of iNOS significantly decreased following MAGL knockdown when compared with the LPS group. In contrast, Arg1 expression significantly increased (P<0.01). As shown in Fig. 4F, compared with the Control group, the proportion of iNOS-positive cells in the LPS group was significantly higher. In contrast, the proportion of Arg1 positive cells was significantly higher than that in the LPS group after MAGL knockdown (P<0.01). The expressions of inflammatory factors IL-1β, TNF-α, and IL-6 were also examined. As shown in Fig. 4G-J, compared with the control group, the expressions of IL-1β, TNF-α, and IL-6 significantly increased after M1 polarization (P<0.01), while MAGL knockdown significantly reduced the expression of IL-1β, TNF-α, and IL-6 compared with the LPS group (P<0.01). Based on the above results, MAGL knockdown suppressed polarization towards an M1 phenotype and promoted polarization towards the M2 phenotype.

MAGL knockdown enhances mitophagy of M1 macrophages in vitro. As shown in the electron microscopy images in Fig. 5A, mitochondria with abnormal morphology with mitophagy were observed in the synovial tissues of patients in the Control and OA groups. Fluorescence imaging and mitophagy protein markers were evaluated further to verify the possible role of MAGL on mitophagy. The mitophagosome is a red fluorescent probe, which is bound to mitochondria in the cell by chemical bonds, while the lysosome is a green fluorescent probe; thus, yellow fluorescence is observed when they are colocalized. The amount of yellow fluorescence represented the levels of mitophagy. As shown in Fig. 5B, there was almost no yellow fluorescence in the Control group, only a little yellow fluorescence in the LPS group, and a considerable amount of yellow fluorescence in the macrophages in the MAGL siRNA group. The PINK1 and Parkin expression levels in macrophages were examined by western blotting. As shown in Fig. 5C-E, compared with the LPS group, the protein expression levels of PINK1 and Parkin in the MAGL siRNA group were significantly increased (P<0.01). The above results indicated that MAGL knockdown by siRNA increased mitophagy levels in M1 macrophages.

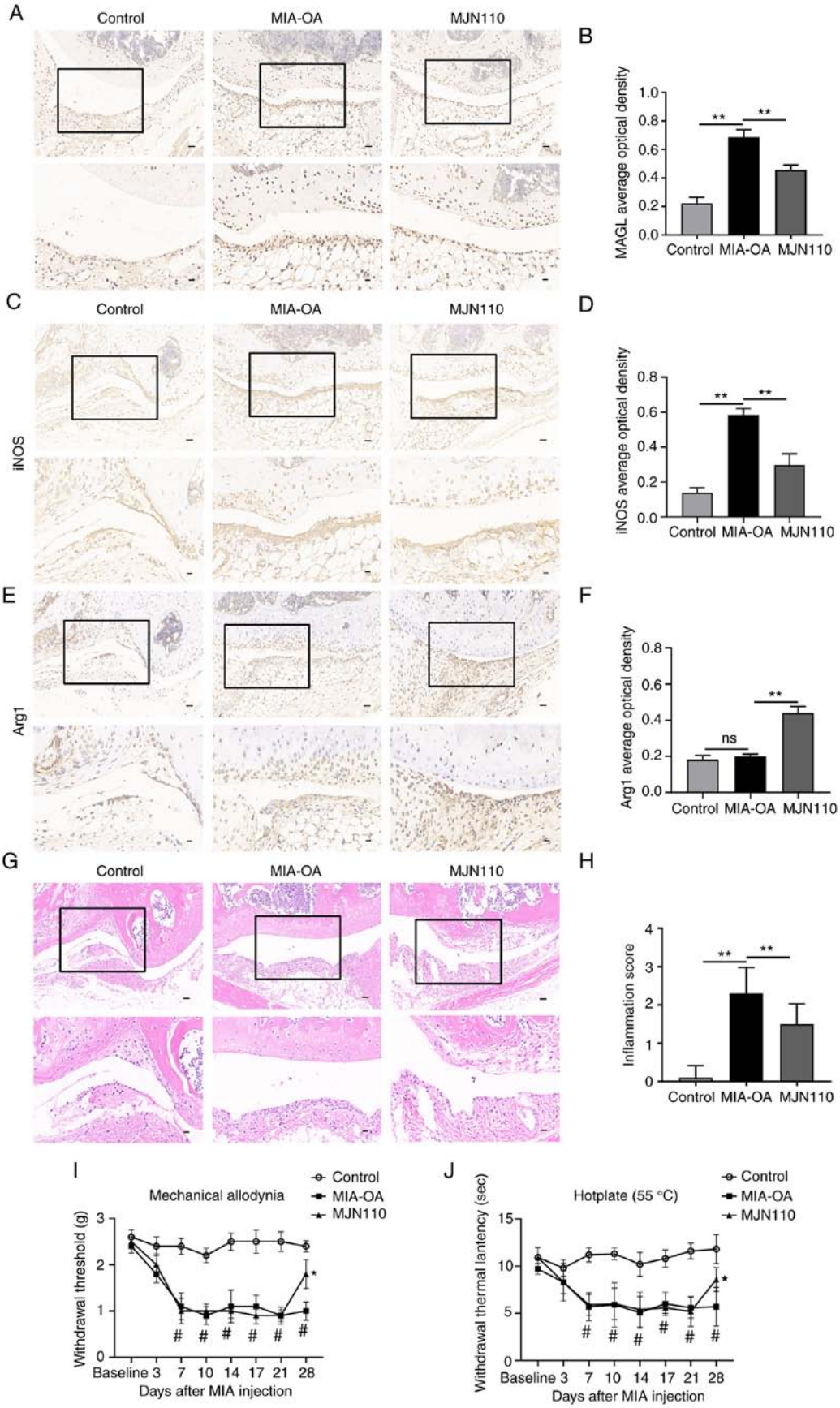

Figure 2. Inhibition of MAGL promotes the polarization of macrophages towards an M2 phenotype and alleviates pain behaviors in OA mice. (A-F) Immunohistochemical staining of MAGL, iNOS, and Arg1 in synovial tissues (upper panel, x200, scale bar 50 μm; lower panel, x400, scale bar 20 μm). (G) Representative images of the synovial tissues stained with H&E upper panel, x200, scale bar 50 μm; lower panel, x400, scale bar 20 μm). (H) Inflammation scores of the synovial tissues. (I) The mechanical pain threshold of mice was measured by electronic von Frey. (J) The thermal pain threshold of mice was measured using a hot plate (n=5 per group). *P<0.05, **P<0.01 vs. MIA-OA group; $^{\#}$P<0.05 vs. control group. ns, not significant; MAGL, monoacylglycerol lipase; OA, osteoarthritis; H&E, hematoxylin and eosin; MIA, monoiodoacetate.

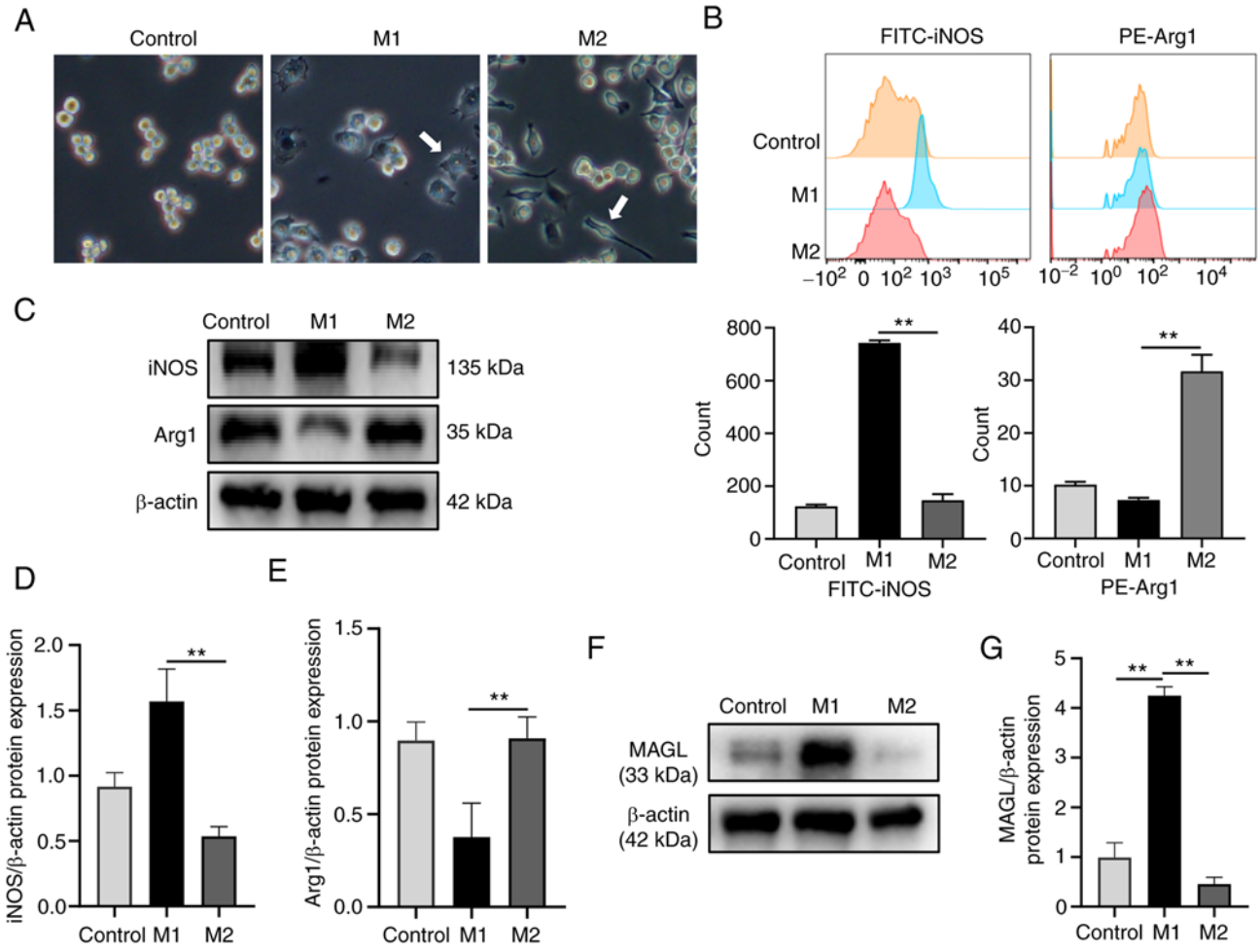

Figure 3. MAGL results in accumulation of M1 polarized macrophages *in vitro*. (A) Representative morphological changes of macrophages in the three groups (white arrow shows a morphologically changed macrophage) Magnification, x100. (B) iNOS and Arg1 expression in the three groups were detected by flow cytometry. (C-E) Western blotting of iNOS and Arg1 in the three groups. (F and G) Western blotting of MAGL in the three groups (n=3 per group). **P<0.01 vs. M1 group. MAGL, monoacylglycerol lipase; iNOS, inducible nitric oxide synthase; Arg1, arginase 1.

Discussion

Knee OA is a common degenerative disease characterized by joint pain and cartilage destruction, which is more common in the elderly. The incidence of OA has increased with the advent of an aging society but is also becoming increasingly diagnosed in the younger population as well (31). Chronic persistent knee joint pain is the primary clinical manifestation of patients with OA of the knee, and it can seriously affect a patient's quality of life. The specific mechanism by which OA pain manifests is unclear. However, it is generally accepted that synovial inflammation caused by the release of inflammatory factors leads to the destruction of the synovium and cartilage is an essential factor of OA pain (32). Therefore, regulating a synovial inflammatory response and reducing the levels of proinflammatory cytokines form the basis for alleviating OA pain.

MAGL regulates the endocannabinoid system by hydrolyzing 2-AG and plays a vital role in inflammatory responses, analgesia, and neuroprotection (11,33,34). In the present study, the role and mechanism of MAGL in OA pain were assessed. It was found that the synovial tissue inflammation score and the expression levels of MAGL increased significantly in OA patients and MIA-induced OA mice. MJN110, a potent pharmacological inhibitor of MAGL, was intraperitoneally injected for 7 consecutive days in OA mice, and this reduced the synovial tissue inflammation score significantly, and the thresholds of mechanical and thermal pain were significantly increased following MAGL inhibition, indicating that MAGL could affect the regulation of OA pain.

Synovial inflammation plays a vital role in the pathological process of OA and is an essential factor in OA pain. The infiltration of inflammatory cells in synovial tissues further aggregates a variety of inflammatory and immune cells, especially macrophages, which are widely involved in the inflammatory cascade (35,36). Macrophages are highly plastic and can polarize towards M1 or M2 phenotypes following specific cues from the environment, and each phenotype exerts contrasting functions. M1 macrophages secrete proinflammatory cytokines such as IL-1β, IL-6, and TNFα, which aggravate the inflammatory response and stimulate nociceptive receptors of peripheral sensory nerves. In contrast, M2 macrophages secrete IL-10 and other anti-inflammatory cytokines, which can reduce the inflammatory response (37,38). However, whether MAGL modulates OA pain by affecting macrophage polarization in synovial tissue requires further study. The present study showed that the synovial macrophages

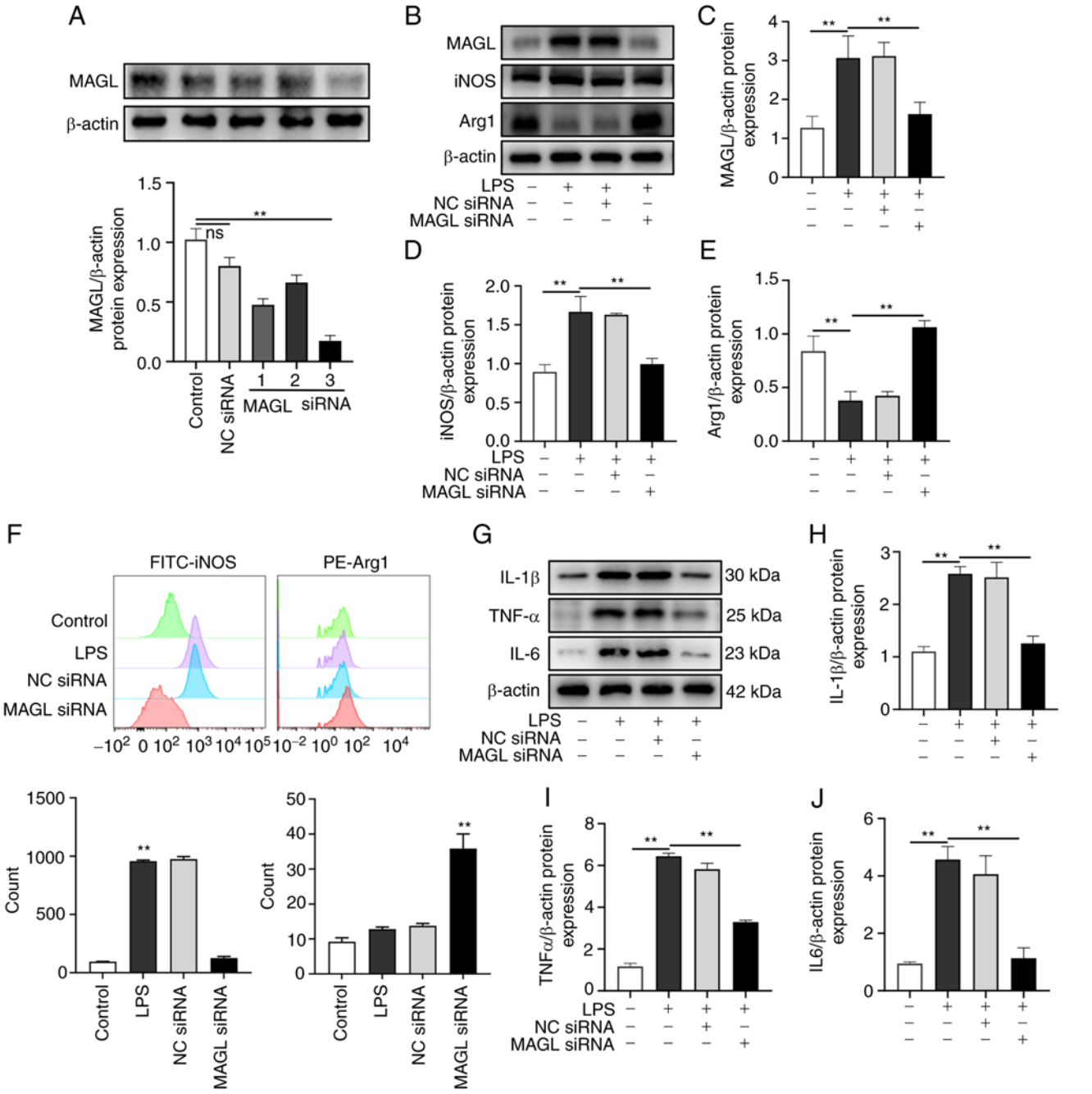

Figure 4. MAGL knockdown promotes M1 to M2 polarization *in vitro*. (A) The protein expression levels of MAGL in macrophages following siRNA transfection (n=3 per group), **P<0.01 vs. control group. (B-E) Western blotting of MAGL, iNOS, and Arg1 in the four groups. (F) iNOS and Arg1 expression levels in the four groups were detected by flow cytometry. (G-J) Western blotting of IL-1β, TNF-α, and IL-6 in the four groups (n=3 per group) **P<0.01 vs. LPS group. MAGL, monoacylglycerol lipase; iNOS, inducible nitric oxide synthase; Arg1, arginase 1.

of OA patients and OA mice were primarily polarized towards an M1 phenotype. The pharmacological inhibition of MAGL by injecting MJN110 showed that MAGL inhibition promoted synovium macrophage polarization from an M1 phenotype towards an M2 phenotype in OA mice. Additionally, double immunofluorescence staining showed that MAGL co-localization with an M1 polarization marker increased in OA patients. However, the mechanism involved in regulating macrophage polarization by MAGL requires further study.

Mitophagy degrades damaged mitochondria in cells and regulates cell metabolism through autophagy, which is critical for cell function and mitochondrial network function, and it plays a vital role in the maintenance of homeostasis and protects nerve cells by removing damaged mitochondria (39-41). In a model of neuropathic pain, it was found that the secretion of proinflammatory cytokines was decreased by enhanced mitophagy levels, which significantly alleviated the response to pain (42). PINK1 and Parkin are important molecules regulating mitophagy and play an essential role in maintaining mitochondrial function. Typically, the expression levels of PINK1 are low, and PINK1 is blocked from entering the inner mitochondrial membrane and thus accumulates on the outer mitochondrial membrane when mitochondria are damaged, and it recruits Parkin to the damaged mitochondria

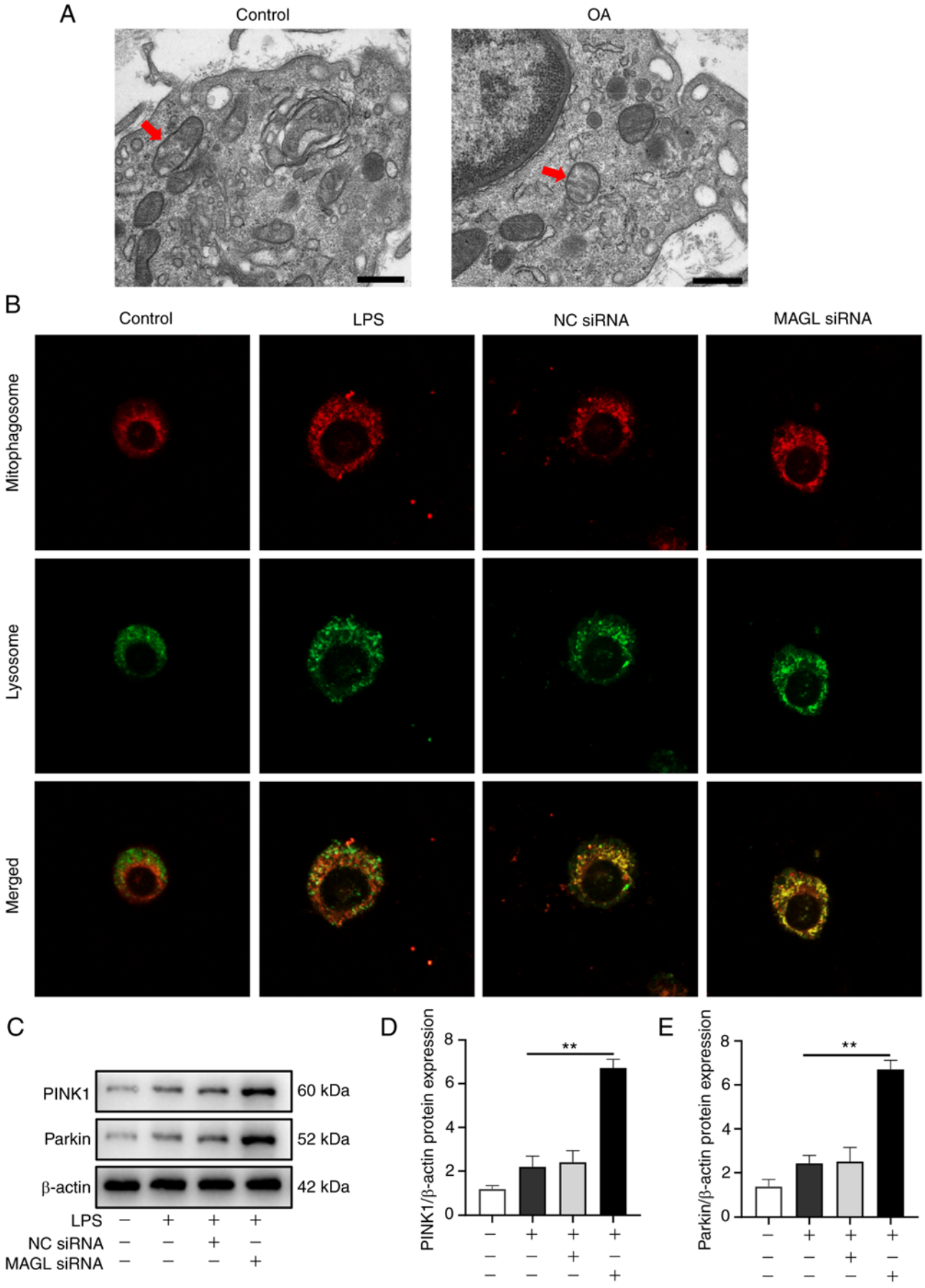

Figure 5. MAGL knockdown enhances the mitophagy of M1 macrophages *in vitro*. (A) Mitochondria with abnormal morphology with mitophagy was observed via electron microscopy in the synovial tissues of patients in the Control and OA groups (red arrow). Scale bar, 5 μm. (B) Immunofluorescence staining of the mitophagosome (red) with the lysosome (green) in the macrophages of the four groups. Magnification, x1000. (C-E) Western blotting of PINK1 and Parkin in the four groups. n=3 per group, **P<0.01 vs. LPS group. MAGL, monoacylglycerol lipase; OA, osteoarthritis.

at the same time (43-46). It was found that after treating cells with Carbonyl Cyanide m-Chlorophenylhydrazine for 3 h to induce mitophagy, increased protein expression of PINK1 was detected (47). In the present study, it was found that mitophagosome and lysosome colocalization significantly increased under confocal microscopy using a mitophagy detection kit in the MAGL siRNA group, and the protein expression levels of PINK1 and Parkin, indicating that mitophagy levels in M1 macrophages increased after MAGL knockdown.

The present study has several limitations. First, the study did not include knockout mice. The gene knockout mice may help further elucidate the role of MAGL in regulating mitophagy and polarization of synovial macrophages. Second, male C57 mice were used to establish the MIA-OA model in these experiments. The role of MAGL in OA pain in aged female mice should thus be also assessed. Third, the detailed molecular mechanism of MAGL inhibiting mitophagy requires elucidation.

In conclusion, the present study demonstrated that MAGL accumulated in the synovial tissues of patients and mice with OA, and inhibition of MAGL promoted the polarization of synovial macrophages from an M1 towards an M2 phenotype and alleviated pain in OA mice. Based on these results, it is proposed that MAGL regulates synovial macrophage polarization by inhibiting mitophagy in OA.

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Availability of data and materials

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

Authors' contributions

CW and DG conceived, designed the study and revised the manuscript. CG and MC performed the experiments, analyzed the data and drafted the manuscript. XL analyzed the data. CG, MC and CW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was performed in accordance with the Ethical Standards of the Declaration of Helsinki and was approved by the Ethics Committee of Suzhou Municipal Hospital (approval no. K-2021-066-K01). All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (approval no. Y20210267).

Patient consent for publication

All patients provided informed consent for publication of their data.

Competing interests

The authors declare no competing interests.

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