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ADAM8 influences the activity of synovial macrophages and fibroblast-like synoviocytes in osteoarthritis

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Osteoarthritis (OA) is the most widely diagnosed form of disabling joint disease, and the polarization of macrophages has been demonstrated to influence OA pathogenesis. The knockdown of ADAM8 (a disintegrin and metalloprotease 8) can suppress OA phenotypes, prompting the present study exploring the potential ability of ADAM8 to regulate fibroblast-like synoviocyte (FLS) phenotypes through its effects on the polarization macrophages, ultimately impacting OA progression. Analyses of protein and mRNA expression were conducted through Western immunoblotting and qPCR, the levels of inflammatory factors were assessed by ELISA. While CCK-8 and lactage dehydrogenase release assays were used to quantify cell proliferation and viability. The migratory and invasive activity of FLS cells was also assessed through wound healing and Transwell approaches. The results revealed that M1 macrophages were found to express high levels of ADAM8, thereby promoting inflammatory cytokine production. Knocking down ADAM8 was sufficient to suppress M1 macrophage polarization, thereby indirectly suppressing FLS proliferative, migratory, and invasive activity.These findings suggest thatADAM8 is a key mediator of OA-related inflammation through its ability to promote pro-inflammatory factor expression within M1 macrophages. ADAM8 was also determined to shape FLS phenotypes through the modulation of the polarization of these macrophages, ultimately influencing the progression of OA.

Keywords: ADAM8 (A disintegrin and metalloprotease 8), Fibroblast-like synoviocytes, Macrophage polarization, Osteoarthritis

Osteoarthritis (OA) is the most widely diagnosed age-related degenerative joint disease, affecting upwards of one-quarter of adults throughout the world such that it is a leading driver of disability and morbidity[1,2]. OA patients present with synovial inflammation, remodeling of the subchondral bone, ectopic osteophyte formation, and degeneration of the cartilage[3,4]. However, OA pathophysiology is not thoroughly understood, with no effective approaches to treating or curing this disease having been established to date[5].

Synovial inflammation is a hallmark of early-stage OA[6], in addition to being closely linked to patient symptoms[7]. In the context of OA synovitis, fibroblast-like synoviocytes (FLS) and macrophages that have been activated secrete inflammatory cytokines, ultimately contributing to the more rapid progression of this disease[8]. Excessive FLS proliferative and migratory activity can culminate in synovial inflammation through the production of a range of inflammatory mediators[9,10]. A growing number of studies have also emphasized the central importance of macrophages as mediators of OA-related synovial inflammation[11-13]. Efforts to clarify the precise mechanisms that underlie synovial macrophage reprogramming from inflammatory M1 to anti-inflammatory M2 phenotypes have the potential to help protect against synovitis and the degeneration of cartilage through consequent effects on FLS phenotypes, thus providing new opportunities to treat OA.

ADAM8 (A disintegrin and metalloproteinase 8) is an ADAM family member that exhibits high levels of OA-related expression in the cartilage[14-16]. ADAM8 is also reportedly expressed at high levels in IL-1β-induced FLS cells and human synovial tissues[17]. The silencing of ADAM8 can reportedly suppress OA phentoypes *in vitro,* while also suppressing OA-related FLS migratory and invasive activity *via* the FSCN1/MAPK axis[18]. M1 and M2 macrophages are in a state of dynamic homeostasis, and the loss of normal M1/M2 balance is observed in the synovial tissue in OA patients, contributing to disease progression[19,20]. The degree to which ADAM8 can impact FLS phenotypes and OA progression through

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its effects on macrophage polarization, however, has yet to be assessed.

Here, ADAM8 was knocked down in murine macrophages, after which murine FLS cells were treated with supernatants harvested from these macrophages to clarify the ability of ADAM8 to modulate FLS phenotypes indirectly through its effects on functional macrophage polarization. Together, these results offer new insights into potential strategies for the more effective management of OA.

Materials and Methods

Cell culture

RAW264.7 murine macrophages (TIB-71, ATCC, VA, USA) were cultured in high-glucose DMEM (11965092; Gibco, CA, USA) with 100 U/mL penicillin, 100 mg/mL streptomycin sulfate (15140122, Gibco), and 10% fetal bovine serum (FBS; 10099141C, Gibco). Murine FLS (CP-M323, Procell, Wuhan, China) were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. All cell culture was performed in a 5% CO_2 incubator at 37°C, with experiments being conducted using cells from passages 4-6.

Macrophage polarization

RAW264.7 cells were treated with LPS (100 ng/mL; Peprotech, USA) or IL-4 (20 ng/mL; Peprotech) for 24 h to respectively induce M1- and M2-like polarization.

Cell transfection

An ADAM8-specific siRNA (si-1, 5′-GCCA-AUCCGGAAUCUGCAUTT-3′; si-2, 5′-GCGUG-CUGUCUCGGAACAATT-3′; si-3, 5′-GCCUA-CCUGCUCAUGGAAUTT-3′) or a corresponding negative control construct (si-NC, 5′-UUCUCCGA-ACGUGUCACGUTT-3′) from GenePharma (Shanghai, China) were transfected into RAW264.7 cells with Lipofectamine 3000 (L3000150, Invitrogen) as directed.

Wound healing assay

Single-cell FLS suspensions from appropriate treatment groups were added to 6-well plates (1×10^6/well), followed by the generation of a scratch wound in the cell monolayer using a 200 μL pipette tip. Following a rinse with PBS, serum-free DMEM was added to the cells, which were then incubated. An inverted microscope (IX71, Olympus, Toyko, Japan) was used for the imaging of cells at 0 and 24 h.

Transwell assay

Transwell inserts pre-treated with Matrigel (Corning Costar, MA, USA) were used for invasion assays. For all Transwell assays, FLS suspensions in serum-free DMEM were added at 2×10^4 cells/well to the upper chamber, whereas the lower chamber was filled with DMEM containing 10% FBS. Following a 48 h incubation, fixation for 20 min using 4% paraformaldehyde was performed, with subsequent staining for 30 min using 0.1% crystal violet, and staining with an inverted microscope (IX71, Olympus, Toyko, Japan).

Analyses of cellular proliferation and death

The proliferative activity of murine FLS was analyzed with a Cell Counting Kit-8 (CCK-8; C0037, Beyotime, Shanghai, China). A CytoTox 96 Non-Radioactive Cytotoxicity assay (G1780, Promega, Madison, WI, USA) was employed to quantify LDH release as a measure of cell death.

qPCR

The qPCR was performed according to previous studies[21, 22]. In brief, TRIzol (15596018CN, Invitrogen, CA, USA) was used as directed to extract cellular RNA, followed by the use of a cDNA Synthesis Kit (FSQ-301, Toyobo, Osaka, Japan) for cDNA preparation and SYBR Premix Ex Taq II (RR820A, TaKaRa, Beijing, China) for qPCR analyses. The $2^{-\Delta\Delta CT}$ method was employed to assess relative expression, with GAPDH as a normalization control. The following primers were used (5'-3'):

ADAM8: GCAGGACCATTGCCTCTACC (forward);
ADAM8: TGGACCCAACTCGGAAAAAGC (reverse);
iNOS: GTTCTCAGCCCAACAATACAAGA (forward);
iNOS: GTGGACGGGTCGATGTCAC (reverse);
CD206: CTCTGTTCAGCTATTGGACGC (forward);
CD206: CGGAATTTCTGGGATTCAGCTTC (reverse);
IL-1β: TTCAGGCAGGCAGTATCACTC (forward);
IL-1β: GAAGGTCCACGGGAAAGACAC (reverse);
IL-4: GGTCTCAACCCCCAGCTAGT (forward);
IL-4: GCCGATGATCTCTCTCAAGTGAT (reverse);
TNF-α: ATGGGAAGGGAATGAATCCACC (forward);
TNF-α: GTCCACATCCTGTAGGGCGTCT (reverse);
IL-6: TAGTCCTTCCTACCCCAATTTCC (forward);
IL-6: TTGGTCCTTAGCCACTCCTTC (reverse);
TGF-β: CTGGATACCAACTACTGCTTCAG (forward);
TGF-β: TTGGTTGTAGAGGGCAAGGACCT (reverse);

IL-10: GCTCCAGAGCTGCGGACT (forward);
IL-10: TGTTGTCCAGCTGGTCCTTT (reverse);
GAPDH: CCCCACACACATGCACTTACC (forward);
GAPDH: CCTACTCCCAGGGCTTTGATT (reverse).

Western immunoblotting

Western immunoblotting analysis was conducted with reference to previous reports[23, 24]. A BCA assay (P0012, Beyotime, Shanghai, China) was used to analyze protein levels in samples extracted using RIPA buffer (P0013E, Beyotime). These proteins were separated by 10% SDS-PAGE, transferred onto PVDF blots (IEVH07850, Millipore, Billerica, USA), and these blots were blocked for 1 h at room temperature (25°C) prior to overnight incubation with antibodies specific for ADAM8 (1:500, ab236949, Abcam, Cambridge, UK); CD206 (1:1000, ab64693, Abcam); iNOS (1:1000, ab178945, Abcam); IL-4 (1:500, AF5142, Affinity, Cincinnati, USA); IL-1β (1:1000, ab234437, Abcam); TNF-α (1:1000, ab183218, Abcam); IL-10 (1:1000, ab310329, Abcam); β-actin (1:1000, GB11001-100, Servicebio, Wuhan, China) at 4°C. After probing at room temperature for 1 h using secondary antibodies (1:2500, FSM0075, FUSHENBIO, Shanghai, China). Bands were visualized with ECL reagents (WBULS0100, Millipore) and analyzed with ImageJ (NIH, Bethesda, MD, USA).

Enzyme linked immunosorbent assay (ELISA)

The amount of RAW264.7 cells secreted IL-6, TNF-α, IL-10, andTGF-β were determined by an ELISA kit (Thermo Scientific, USA), according to the manufacturer's instruction.

Statistical analyses

Analyses were conducted using GraphPad Prism 9.5 (GraphPad Software Inc., CA, USA). Results were presented as means ± standard deviation (SD) and compared with Student's t-tests or one-way ANOVAs, with $P< 0.05$ as the threshold of significance.

Result

ADAM8 expression is highly correlated with the polarization of macrophages

An initial analysis of the relationship between ADAM8 and the polarization of macrophages was conducted by exploring any potential polarization-related changes in the expression of this protein. While the induction of M1-like differentiation led to significant increases in the protein and mRNA levels of ADAM8 within macrophages, the opposite was observed in the context of M2-like differentiation (Fig. 1A-C). This suggested that ADAM8 may play a role as a regulator of OA through its effects on macrophage polarization.

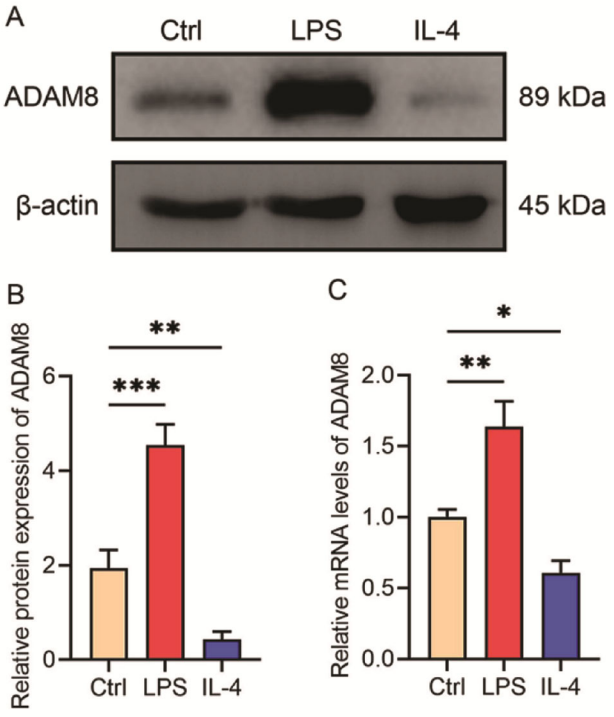

Fig. 1 — Analyses of the expression of ADAM8 in polarized macrophages. (A-C) Western immunoblotting and qPCR were used to assess ADAM8 protein (A & B) and mRNA (C) levels in RAW264.7 cells following IL-4 or LPS stimulation. Data are means ± SD (n = 3). *$P< 0.05$, **$P< 0.01$, ***$P< 0.001$. Ctrl, control; LPS, lipopolysaccharide; IL-4, interleukin-4

ADAM8 promotes M1 and suppresses M2 macrophage polarization

RAW264.7 cells were treated with LPS to establish an *in vitro* OA cell model, and OA-like phenotypes were detected in this cell model. As shown in Figure 2A, ELISA results indicated that the production of TNF-α and IL-6 was found to stimulated in LPS-treated RAW264.7 cells compared to control cells. Next, to clarify how ADAM8 affects polarized macrophage phenotypes, an ADAM8-specific siRNA was transfected into RAW264.7 cells, followed by analyses of M1 (iNOS) and M2 (CD206) markers. Western immunoblotting and qPCR confirmed ADAM8 knockdown efficacy, with the greatest silencing having been achieved for si-ADAM8-1 such that it was used in the following experiments (Fig. 2B-D). Following LPS treatment, iNOS protein and mRNA levels rose

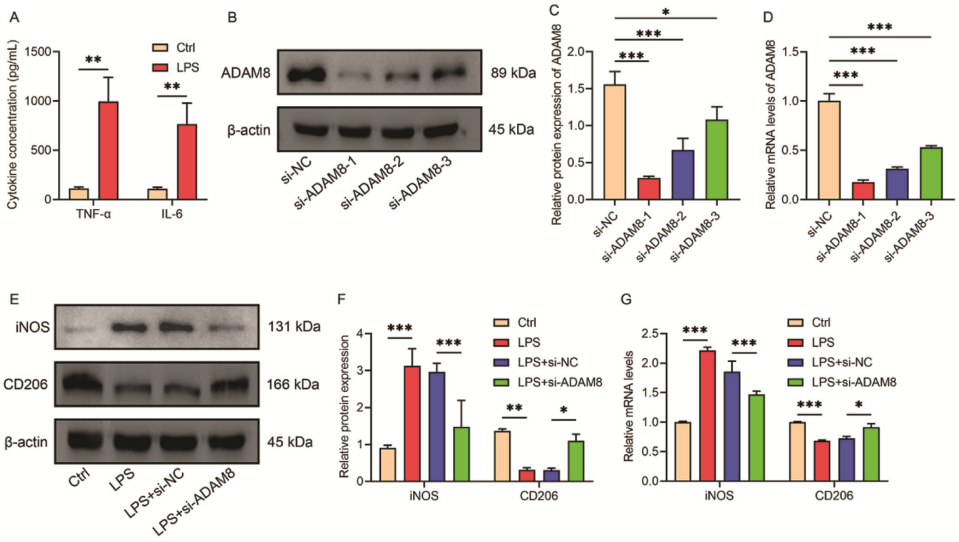

Fig. 2 — Knocking down ADAM8 promotes the M2 polarization of macrophages. (A) The protein level of TNF-α and IL-6 were measured by ELISA. (B-D) Following transfection with ADAM8-targeting siRNAs, ADAM8 levels in HLF cells were analyzed *via* Western immunoblotting (B & C) and qPCR (D); (E-G) iNOS and CD206 protein (E & F); mRNA (G) levels were analyzed in the indicated groups. Data are means ± SD (n = 3). **P*< 0.05, ***P*< 0.01, ****P*< 0.001. TNF-α, tumor necrosis factor alpha; IL-6, interleukin-6; si-NC, negative control siRNA; si-ADAM8-1/2/3, siRNAs specfic for ADAM8; Ctrl, control; LPS, lipopolysaccharide; si-ADAM8, siRNA specific for ADAM8

significantly in RAW264.7 cells, while corresponding decreases in CD206 protein and mRNA expression were observed. Silencing ADAM8 reversed these changes, lowering iNOS induction while enhancing the expression of CD206 under these conditions (Fig. 2E-G). The silencing of ADAM8 thus appears to favor the anti-inflammatory M2-like polarization of RAW264.7 macrophages relative to their M1-like polarization.

Knocking down ADAM8 inhibits macrophage proinflammatory cytokine expression

Western immunoblotting revealed elevated IL-1β and TNF-α protein levels together with low levels of anti-inflammatory IL-4 and IL-10 in LPS-treated RAW264.7 cells. The silencing of ADAM8 reversed these changes (Fig. 3A-D). The ELISA results showed that ADAM8 knockdown reversed the increase of the pro-inflammatory cytokines secreted by M1 macrophages (TNF-α and IL-6) and decrease of the anti-inflammatory cytokines secreted by M2 macrophages (TGF-β and IL-10) induced by LPS (Fig. 3C). Similar results were observed through qPCR, with LPS treatment being associated with increased IL-1β, TNF-α, and IL-6expression and reduced IL-4, TGF-β, and IL-10expression, while knocking down ADAM8 suppressed IL-1β, TNF-α, and IL-6expression and increased IL-4, TGF-β, and IL-10 expression in these cells (Fig. 3D). These data suggest that knocking down ADAM8 silencing suppresses macrophage proinflammatory cytokine expression.

Knocking down ADAM8 suppresses FLS proliferation through effects on macrophage polarization

To examine the effects of ADAM8 knockdown on FLS cell proliferative activity and death, murine FLS cells were treated with supernatants harvested from macrophages in which ADAM8 had been silenced. In a CCK-8 assay, LPS-induced FLS cell proliferation was significantly suppressed in this assay when ADAM8 was knocked down (Fig. 4A). Consistently, significantly increased cell death was observed after the knockdown of ADAM8 relative to the LPS treatment group (Fig. 4B). ADAM8 knockdown can thus suppress FLS cell proliferation and induce the death of these cells through its effects on the polarization of macrophages.

Knocking down ADAM8 inhibits FLS cell migration and invasivity through effects on the polarization macrophages

Cellular invasion and migratory activity were next analyzed to examine the impact of ADAM8 on these activities in FLS cells *via* its effects on the polarization of macrophages. Wound healing assays (Fig. 5A & B) and Transwell migration/invasion assays (Fig. 5C & D) revealed that LPS stimulation induced significant FLS cell migratory and invasive activity, whereas ADAM8 knockdown significantly

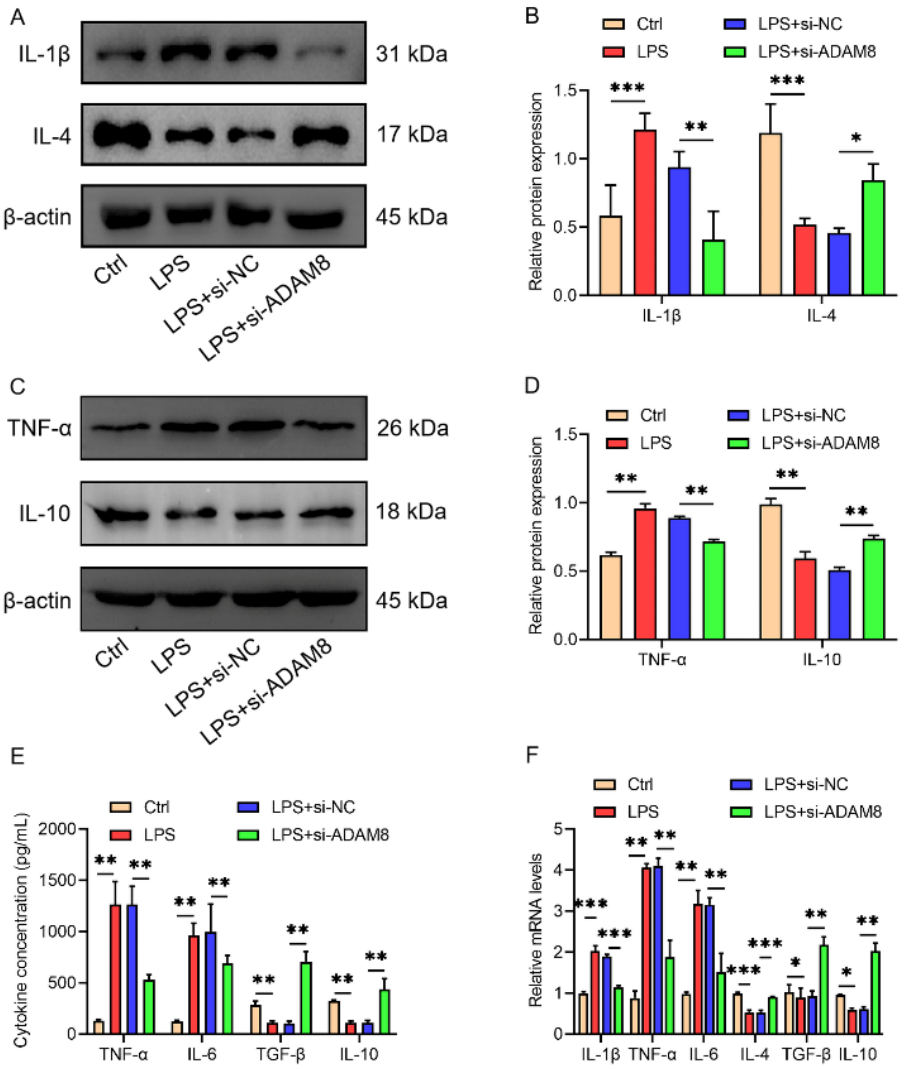

Fig. 3 — Knocking down ADAM8 inhibits proinflammatory cytokine levels in macrophages. (A-D) The expression of IL-1β, IL-4, TNF-α, and IL-10 was analyzed *via* Western immunoblotting. (E) The protein level of TNF-α, IL-6, TGF-β, and IL-10 were measured by ELISA. (F) The mRNA level of IL-1β, TNF-α, IL-6, IL-4, TGF-β, and IL-10 were measured by qPCR. Data are means ± SD (n = 3). **P*< 0.05, ***P*< 0.01, ****P*< 0.001. Ctrl, control; LPS, lipopolysaccharide; si-NC, negative control siRNA; si-ADAM8, siRNA specific for ADAM8; IL-β, interleukin beta; IL-4, interleukin-4; TNF-α, tumor necrosis factor alpha; IL-6, interleukin-6; TGF-β, transforming growth factor beta; IL-10, interleukin-10

suppressed such activity. These data indicate that the silencing of ADAM8 can inhibit FLS cell migratory and invasive activity in FLS cells through effects on the polarization of macrophages.

Discussion

As a chronic disease that generally presents with synovitis, OA can cause prominent symptoms including arthralgia and impaired function[25]. This study was conducted to explore how ADAM8 functions in OA. In these analyses, higher ADAM8 expression was noted in M1 macrophages wherein it was found to support inflammatory cytokine release. When FLS cells were cultured in the presence of supernatants derived from si-ADAM8-transfected macrophages, this led to the induction of phenotypic changes in these cells. Together, these results offer novel insights regarding the applicability of ADAM8 in the context of OA treatment, suggesting that efforts to target macrophage polarization and macrophage interactions with FLS cells may represent an effective means of preventing OA progression or treating affected individuals.

The ADAM family protein ADAM8 has been widely studied in oncology settings[26-28], wherein it has most often been found to favortumor progression and inflammatory activity[29]. Zack *et al.*[16] determined that ADAM8 is capable of cleaving fibronectin to facilitate OA development. However, more remains to be discovered with respect to how ADAM regulates the pathogenesis of OA. Here, significantly higher ADAM8 expression was noted in M1-like macrophages, consistent with its potential importance in the context of OA progression.

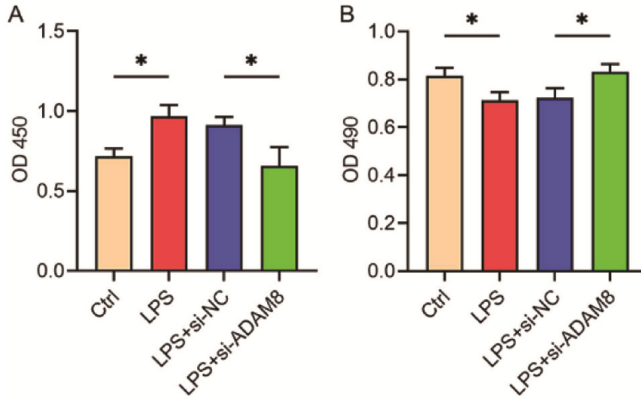

Fig. 4 —Knocking down ADAM8 suppresses FLS cell proliferation through impacts on the polarization of macrophages. (A) Cellular viability was analyzed *via* CCK-8 assay; and (B) A CytoTox 96 Non-Radioactive Cytotoxicity assay was used to assess cell viability. Data are means ± SD (n = 3). **P*< 0.05. Ctrl, control; LPS, lipopolysaccharide; si-NC, negative control siRNA; si-ADAM8, siRNA specific for ADAM8

OA is characterized by a range of pathological alterations in specific types of cells, including FLS and macrophages present in the synovial tissue[30]. Macrophages are key regulators of OA-related inflammation[31], and the dysregulation of M1/M2 homeostasis has been suggested to be potentially important as a component of OA progression[32]. through their ability to secrete inflammatory factors, M1 macrophages can induce OA-related pain, whereas M2 macrophages are responsible for the release of anti-inflammatory mediators. Zhang *et al.*[33] demonstrated that macrophages exhibiting M1 polarization accumulate within the synovium in both mice and humans in the context of OA. Here, RAW264.7 cells were induced to undergo M1- or M2-like polarization, with subsequent analyses revealing high levels of ADAM8 expression in the

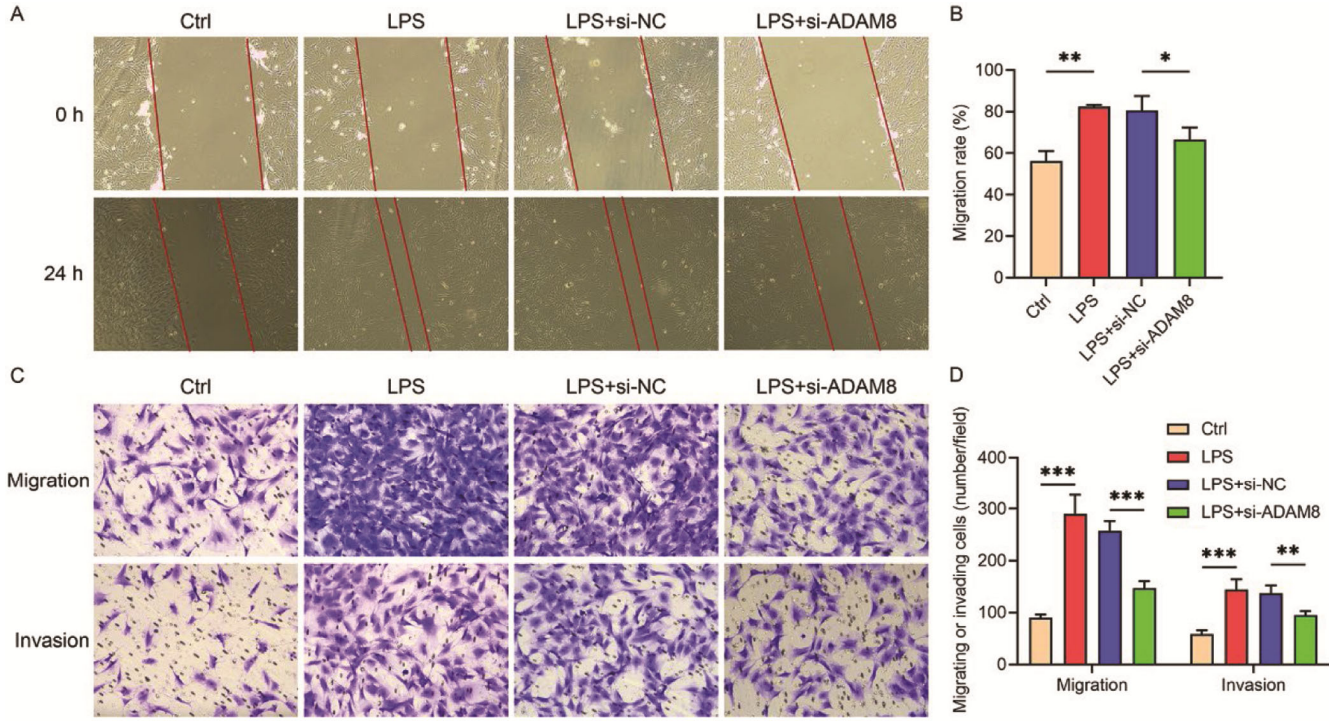

Fig. 5—Knocking down ADAM8 inhibits FLS cell invasion and migratory activity through effects on macrophage polarization. (A & B) Migratory distance was analyzed at 0 and 24 h *via* wound healing assay (n = 3); and (C & D) Transwell migration and matrigel invasion analyses were performed in the indicated groups in which cells that were adherent to the membrane were photographed and enumerated. Magnification ×200. Data are means ± SD (n = 6). **P*< 0.05, ***P*< 0.01, and ****P*< 0.001. Ctrl, control; LPS, lipopolysaccharide; si-NC, negative control siRNA; si-ADAM8, siRNA specific for ADAM8

M1-like cells whereas its expression in M2-like cells was limited. To test the functional role of ADAM8 in the context of macrophage polarization, it was knocked down in LPS-treated murine macrophages through an siRNA-based approach, which ultimately revealed that ADAM8 was able to promote iNOS expression while suppressing CD206. When ADAM8 was silenced, inflammatory IL-1β, TNF-α, and IL-6 levels declined whereas anti-inflammatory IL-4, TGF-β, and IL-10 levels increased in macrophages, consistent with its role as a mediator of M1 polarization. Mesenchymal FLS are widely distributed throughout the synovial tissue, and in OA patients these cells exhibit enhanced migratory and invasive activity[34]. Silencing ADAM8 in OA has been reported to suppress FLS cell migration and invasivity[18]. NOD2 overexpression can further limit the migratory and invasive activity of FLS cells through its ability to attenuate M1 macrophage polarization[34]. Here, an experiment in which supernatants from ADAM8-knockdown macrophages were cultured with FLS cells revealed ADAM8-dependent changes in FLS cell phenotypes, in line with prior reports from Chen *et al*.[18]. The present study is distinct, however, in that it focused on the mechanism through which ADAM8 can affect the differentiation of macrophages to indirectly alter FLS outcomes. These findings demonstrate the important intermediate role that macrophages play in the pathogenesis of OA while also unveiling the link between ADAM8 expression in macrophages and FLS phenotypes, ultimately leading to altered OA progression.

Despite the promising results, this study has several limitations. Notably, the experiments were conducted *in vitro*, and the lack of *in vivo* validation poses a significant constraint. The *in vivo* environment presents complexities and interactions that are not fully replicable *in vitro*, and thus, further studies using animal models are necessary to confirm these findings. Future research should aim to address these limitations by incorporating *in vivo* models to validate the role of ADAM8 in OA progression and explore its potential as a therapeutic target.

Conclusion

In summary, M1 macrophages exhibit high levels of ADAM8 expression, with this ADAM family member serving as an important promoter of M1 polarization and inflammatory mediator production. ADAM8 was also capable of promoting FLS migration, proliferation, and invasivity by means of its effects on the polarization of macrophages, thereby driving accelerated OA progression.

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Conflicts of interest

All authors declare no conflicts of interest.

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