

Semaphorin 6D Alleviates Osteoarthritis by Inhibiting NLRP3 Inflammasome Activation and Endoplasmic Reticulum Stress

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Objective: Osteoarthritis (OA) is a degenerative joint disease that affects over 500 million individuals globally, characterized by the degradation of cartilage, subchondral bone sclerosis, and synovitis. A key factor in the progression of OA is synovial inflammation, which is driven by macrophage polarization and inflammasome activation. This inflammation exacerbates cartilage degradation, creating a vicious cycle that accelerates disease progression. Targeting macrophage activity presents a promising therapeutic strategy to alleviate the symptoms and progression of OA.

Methods: To investigate the role of SEMA6D in macrophage polarization and its potential therapeutic implications for OA, we conducted transcriptomic analysis to explore its regulatory functions. In vitro experiments were performed to assess the effects of SEMA6D overexpression on the expression of ASC and NLRP3, as well as on macrophage (RAW264.7) polarization toward the pro-inflammatory M1 phenotype. In vivo studies were conducted using an OA rat model to evaluate the influence of SEMA6D overexpression on synovial macrophage polarization and the levels of inflammatory mediators, including IL-1 β and IL-6.

Results: Our transcriptomic analysis indicated that SEMA6D plays a regulatory role in macrophage polarization. Overexpression of SEMA6D resulted in the downregulation of ASC and NLRP3, effectively inhibiting the polarization of macrophages toward the M1 phenotype and downregulate the expression levels of iNOS and IL-6 by more than twofold. In the DMM rat model, SEMA6D overexpression significantly reduced the polarization of synovial macrophages to the M1 phenotype, leading to lower levels of inflammatory mediators such as IL-1 β and IL-6, and mitigating cartilage degeneration.

Conclusion: SEMA6D exerts a protective effect against OA by attenuating synovial macrophage-mediated inflammatory responses through the inhibition of NLRP3 inflammasome activation and endoplasmic reticulum stress.

Keywords: osteoarthritis, NLRP3, inflammasome, SEMA6D, macrophage

Introduction

Osteoarthritis (OA), a degenerative joint disease, is becoming increasingly prevalent, affecting more than 500 million people worldwide.¹ OA is characterized by the degeneration of articular cartilage, subchondral bone sclerosis, and synovitis.^{2,3} The pain and limited joint function caused by OA significantly impair patients' quality of life and place a substantial burden on healthcare systems.^{4,5} Therefore, identifying effective therapeutic strategies for OA is of critical importance. Synovitis is believed to play a key role in the progression of OA. The release of inflammatory mediators

from the synovium exacerbates cartilage matrix degradation, which in turn recruits more inflammatory cells, forming a vicious cycle.^{6,7} Hence, ameliorating synovial inflammation is expected to have a positive impact on OA treatment.

The synovium consists of two distinct layers: the intimal lining layer, composed of macrophages and synoviocytes, and the sublining layer, made up of connective tissue, blood vessels, and a small number of lymphocytes.⁸ During OA progression, cartilage fragments and intracellular proteins from necrotic cells act as danger-associated molecular patterns (DAMPs) to activate synovial macrophages, subsequently triggering downstream intracellular signaling pathways such as NLRP3 inflammasome activation.^{9–12} The activation of inflammasomes induces the maturation and secretion of IL-1 β and IL-18, thereby exacerbating synovial inflammation.^{13–15} Consequently, a deeper understanding of macrophage responses to inflammation and the identification of suitable therapeutic targets are promising approaches to alleviating synovitis and delaying OA progression.

Semaphorin 6D (SEMA6D), a member of the Semaphorin family, is a key factor mediating nervous system development. Recently report, SEMA6D has also been implicated in various physiological processes and disease progression, including the development of the visual system,^{16,17} breast cell proliferation and migration,¹⁸ and cancer progression.^{19–22} Hirai et al found that SEMA6D forward signaling, which is coordinating the orientation of cell development and migration as a guidance factor, impaired the infiltration and activation of tumor-specific CD8+ T cells in murine oral tumors.²³ Nakanishi et al found that SEMA6D coordinates angiogenic, metabolic, and inflammatory outputs from the amygdala by maintaining synaptic homeostasis.²⁴ Duan et al SEMA6D may serve as a diagnostic and prognostic biomarker for clear cell renal cell carcinoma.²² In previous studies, we identified SEMA6D as a negative regulator of miR-7 and demonstrated its role in modulating cartilage metabolism, thus significantly alleviating OA progression.²⁵ However, the mechanism by which SEMA6D regulates the physiological functions of immune cells involved in the progression of OA has not been studied. This prompted us to investigate the role of SEMA6D in the inflammatory response of synovial macrophages.

In this study, we first performed transcriptomic sequencing to examine gene expression profiles in macrophages transfected with pcDNA3.1-SEMA6D (to overexpress SEMA6D) or siRNA-SEMA6D (to silence SEMA6D). The results revealed that SEMA6D is involved in macrophage polarization, with its overexpression suppressing the activation of NLRP3 and TNF α signaling pathways. Further in vitro experiments demonstrated that SEMA6D downregulated the expression of ASC and NLRP3, thereby inhibiting macrophage polarization toward the M1 phenotype and reducing the secretion of inflammatory cytokines. This finding was further validated in an OA rat model, where SEMA6D was shown to suppress synovial macrophage polarization to the M1 phenotype, reduce the levels of inflammatory mediators such as IL-1 β and IL-6, and mitigate cartilage degeneration, thereby alleviating the progression of OA (Scheme.1).

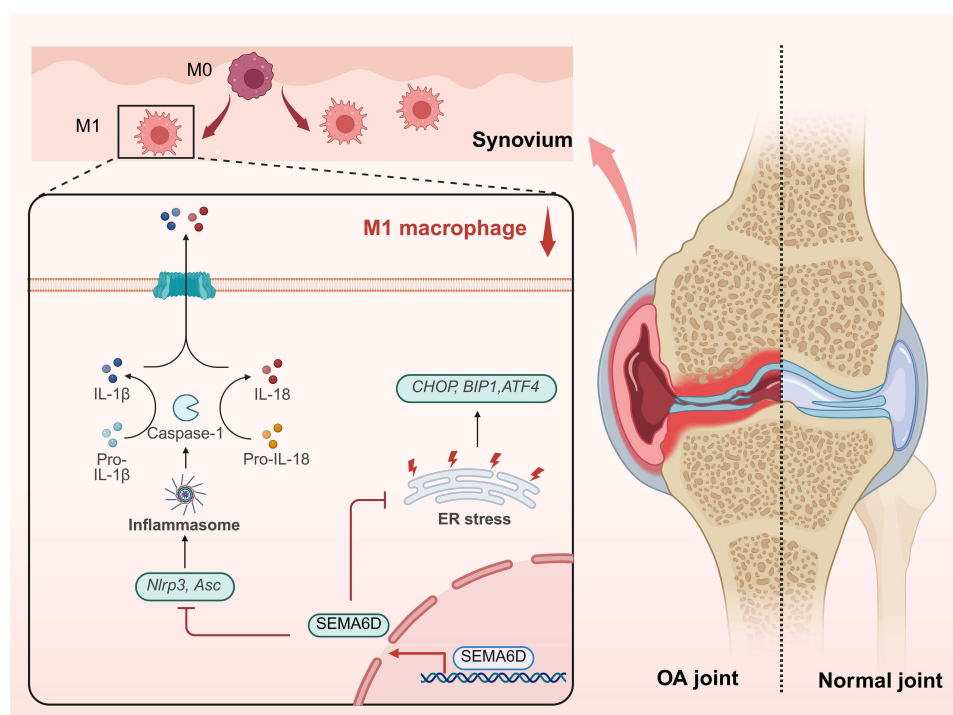
Methods and Materials

Materials and Antibody

DMEM culture medium and fetal bovine serum were purchased from Gibco. The antibodies used in this study are as follows: CD68 (ab53444, Abcam, UK), iNOS (18985-1-AP, Proteintech, USA), NLRP3 (30,109-1-AP, Proteintech, USA), ASC (ab309497, Abcam, UK), CD80 (66,406-1-Ig, Proteintech, USA), IL-1 β (ab283818, Abcam, UK), IL-6 (ab290735, Abcam, UK), SEMA6D (SC-393258, Santa Cruz, USA), ATF4 (10,835-1-AP, Proteintech, USA), BIP-1 (ab21685, Abcam, UK), CHOP (15204-1-AP, Proteintech, USA), IRE1 α (ab37073, Abcam, UK), Caspase-1 (ab238972, Abcam, UK), COX-2 (27,308-1-AP, Proteintech, USA), β -actin (66009-1-Ig, Proteintech, USA).

Cell Culture

The mouse monocyte/macrophage cell line RAW264.7 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were incubated at 95% humidity and 5% CO₂. When the cells reached 80–90% confluence, they were passaged.



Scheme 1 SEMA6D alleviates the progression of OA by inhibiting the activation of the NLRP3 and ASC inflammasomes, while simultaneously reducing endoplasmic reticulum stress, which together decrease the M1 polarization of macrophages.

Cell Transfections

The pcDNA3.1 plasmid and siRNA were purchased from Ribo Bio (China). RAW264.7 cells were seeded in 6-well plates and transfected using Lipo8000™ transfection reagent (Beyotime, China) according to the manufacturer's instructions. Transfection was performed when the cells reached 60–70% confluence. Forty-eight hours post-transfection, target gene expression was assessed by Western blot to evaluate transfection efficiency.

RNA Sequencing

The RAW264.7 cells were divided into two groups: OE-SEMA6D (pcDNA3.1 - overexpression) and Si-SEMA6D (siRNA - knockdown). Total RNA was extracted using trizol reagent (Invitrogen, USA), and gene expression profiling was analyzed through sequencing. Differentially expressed genes (DEGs) were identified using the “clusterProfiler” package in R, with the criteria set as fold change ≥ 2 and adjusted p-value < 0.05 . Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed to explore the relevant biological pathways.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA from RAW264.7 cells was extracted using trizol reagent (Invitrogen). Reverse transcription of equal amounts of RNA was performed using a reverse transcription kit (Applied Biosystems). qRT-PCR was conducted according to the manufacturer's instructions using SYBR Green PCR MasterMix (Applied Biosystems). The relative expression levels of each gene were assessed using the $2^{-\Delta\Delta Ct}$ method, with β -actin expression used for normalization. The primers are shown in Table 1.

Flow Cytometry

RAW264.7 were diluted and seeded in 6-well plates. When the cells were adherent and had grown to the logarithmic phase, the cells were treated with LPS (100 ng/mL) and IFN- γ (20 ng/mL) for 24 h. Subsequently, the level of CD86 in cell were measured using a BD Cytometric Bead Array (CBA) flow cytometry (CyFlow Cube 6 2L4C).

Table 1 qPCR Primer Sequences for Target Gene

Primers	Forward (5'–3')	Reverse (5'–3')
Mus-IL6	GTCCTTCTACCCCAATTCCA	TAACGCACTAGGTTTGCCGA
Mus-TNF- α	AGGCACTCCCCAAAAGATG	TTTGCTACGACGTGGGCTAC
Mus-CD206	ACGAGCAGGTGCAGTTTACA	ACATCCCATAAGCCACCTGC
Mus-YMI	AGCAGAAGCTCTCCAGAAGCAA	ATTGGCCTGTCTTAGCCCA
Mus-fizz1	ACTATGAACAGATGGGCCTCC	AACGAGTAAGCACAGGCAGT
Mus-iNOS	CAACAGGGAGAAAGCGCAA	ATTCTGTGCTGTCCCAGTGAG
Mus-BIP-1	TCGATACTGGCCGAGACAAC	CGACGGTTCTGGTCTCACAC
Mus-ATF4	CCTATAAAGGCTTGCGGCCA	GCTGGATTTCTGTAAGAGCG
Mus-CHOP	TACTGGCTCCGTCTAACCCT	CTGCTTTCAGGTGTGGTGGT
Mus-NLRP3	GTACCCAAGGCTGCTATCTGG	GGACACTCGTCATCTTCAGCA
Mus-IL-1 β	TGCCACCTTTTGACAGTGATG	ATGTGCTGCTGCGAGATTTG
Mus-SEMA6D	GCAAGCCGTCAGGGTGAG	CAAAGTTGCGGGGGAGAGT
Mus- β -actin	ATGTGGATCAGCAAGCAGGA	AAGGGTGAAAACGCAGCTCA

Enzyme-Linked Immunosorbent Assay

IL-6 ELISA Kit (KE10007, Proteintech, Proteintech, USA) and IL-1 β ELISA Kit (KE10002, Proteintech, USA), directed by the manufacturer, were used to quantify IL-6 and IL-1 β in the supernatant of RAW264.7 cells (6-well plates, approximately 6×10^5 cells/well) respectively. Absorbances at 450 nm were read in a microplate reader (ThermoFisher, USA).

Western Blot Analysis

The total proteins in the RAW 264.7 cells of the different groups were lysed using RIPA lysis buffer and extracted. The bicinchoninic acid (BCA) protein assay kit (P0010S, Beyotime) was used for measuring protein concentrations. Each protein sample underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and washed with TBST. The membranes were incubated with primary antibodies overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibodies for two hours at 37°C. Finally, the proteins were visualized by chemiluminescence autoradiography (Thermo Fisher Scientific). The gel pattern is scanned through the gel scanner.

Immunofluorescence Staining

RAW 264.7 cells were seeded into 24-well plates at a density of 1×10^4 /well and cultured for 24 hours to reach 70% confluence. The cells were divided into four groups: the control group, the LPS + IFN- γ group, the LPS + IFN- γ + pcDNA3.1-SEMA6D group, and the LPS + IFN- γ + siRNA-SEMA6D group. RAW264.7 cells were transfected for 24 hours, followed by stimulation with LPS + IFN- γ for 24 hours. Next, the cells were fixed with 4% paraformaldehyde at 37°C for 15 minutes and permeabilized with 0.5% Triton X-100 for 20 minutes. After blocking with 5% BSA for 30 minutes, the cells were incubated overnight at 4°C with primary antibodies: CD68 (ab53444, Abcam, UK), iNOS (18985-1-AP, Proteintech, USA), NLRP3 (30,109-1-AP, Proteintech, USA), and ASC (ab309497, Abcam, UK). The following day, the cells were incubated with a goat anti-rabbit secondary antibody in the dark for 2 hours and mounted using an anti-fluorescent quenching mounting medium containing DAPI staining solution. The images were captured using a fluorescence microscope.

Biological Transmission Electron Microscopy (BTEM)

Cell integrity and changes in organelles were observed using transmission electron microscopy. The cell samples were fixed with precooled 2.5% glutaraldehyde and imaged under a BTEM (HT7800, HITACHI).

Animal Model

Sprague-Dawley (SD) rats were purchased from Cavens Laboratory Animal Co., Ltd. (Changzhou). A total of 15 male SD rats, aged 8 to 10 weeks, with a body weight of approximately 300 to 350 g, were used. The rats were randomly divided into three groups using a random number table: Sham group (sham surgery, saline injection, $n = 5$), Control group (OA model + saline injection, $n = 5$), and AVV6-SEM6D group (OA model + AVV6-SEM6D treatment (1E + 11VG), $n = 5$). After one week of adaptive feeding, OA models were established in 10 rats from the Control and AVV6-SEM6D groups using the medial meniscal destabilization (DMM) surgery,^{26,27} while 5 rats in the Sham group only underwent joint capsule incision and direct suturing. All procedures used in this study were conducted in accordance with the guidelines set by the Chinese Laboratory Animal Management Authority and the National Institutes of Health (NIH). All surgical procedures and perioperative management adhered to Chinese laws and local ethics committee regulations. The animal experiments were approved by the Ethics Committee of the Zhejiang University Animal Experiment Center.

Histological Analysis

The complete knees were fixed with 4% PFA (paraformaldehyde) for 48 h, then the joints were decalcified for 2 months in a 10% EDTA solution and paraffin-embedded. Synovial thickness and inflammation grade were assessed using hematoxylin and eosin (HE) staining (Servicebio Technology, Wuhan, China); the degree of articular cartilage degeneration was assessed using Safranin O-fast green staining (Servicebio Technology, Wuhan, China) combined with the OARSI score, and the degree of synovial fibrosis was assessed using Masson staining (Servicebio Technology, Wuhan, China). Images were acquired using an ordinary optical microscope. To determine M1 macrophage polarization and inflammation, the synovium was stained with immunofluorescence and immunohistochemistry.

Quantification and Statistical Analysis

All data are expressed as the mean \pm SEM. Statistical analyses were performed with GraphPad Prism V.7.0 software. All the data were performed the Shapiro–Wilk test to confirm their distribution. For the normally distributed data, unpaired Student's *t*-test analyzed the differences between two groups of data, and differences among three or more groups were analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA followed by a post hoc Tukey's test. For the non-normally distributed data, the Mann–Whitney test was used for means comparison between the two groups. The Kruskal–Wallis test was used for the means comparison of multiple individual data sets. The Spearman's rank-order correlation test was used for Pearson's correlation analysis. A value of $p < 0.05$ was considered statistically significant. The sample size ranges from 3 to 5 according to the effect of a 50% increase and 25% SD compared with the control group. The acceptable error rate is 5%. Stars indicated significance level: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

The Upregulation of SEMA6D Inhibits the Polarization of M1 Macrophages and Downregulates the Expression of Endoplasmic Reticulum Stress-Related Genes

Our previous study demonstrated that SEMA6D plays a pivotal role in the metabolic regulation of arthritic chondrocytes,²⁵ which may attenuate cartilage matrix degradation and subsequently mitigate the progression of arthritis. To investigate this, we treated RAW264.7 cells with siRNA-SEMA6D and pcDNA3.1-SEMA6D, respectively, and analyzed the effects of SEMA6D on macrophage function through transcriptomic studies. As shown in [Figure 1A](#), compared to the pcDNA3.1-SEMA6D group, 213 genes were upregulated and 315 genes were downregulated following siRNA-SEMA6D treatment. Protein interaction analysis revealed that genes associated with macrophage polarization, such as *Cdc20*, *Plk1*, and *Ccnb1*, played pivotal roles in the network ([Figure 1B](#)). Furthermore, the differentially expressed genes were primarily related to intracellular metabolic processes, and KEGG analysis indicated that inflammation-related pathways, such as the NOD-like receptor signaling pathway and TNF signaling pathway, were activated ([Figure 1C and D](#)). These findings suggest that downregulation of SEMA6D may promote macrophage polarization towards a pro-inflammatory phenotype. Next, we examined macrophage phenotype-related markers using qRT-PCR. In the siRNA-SEMA6D group, the expression of iNOS and IL-6 exceeded that of the pcDNA3.1-SEMA6D group by more

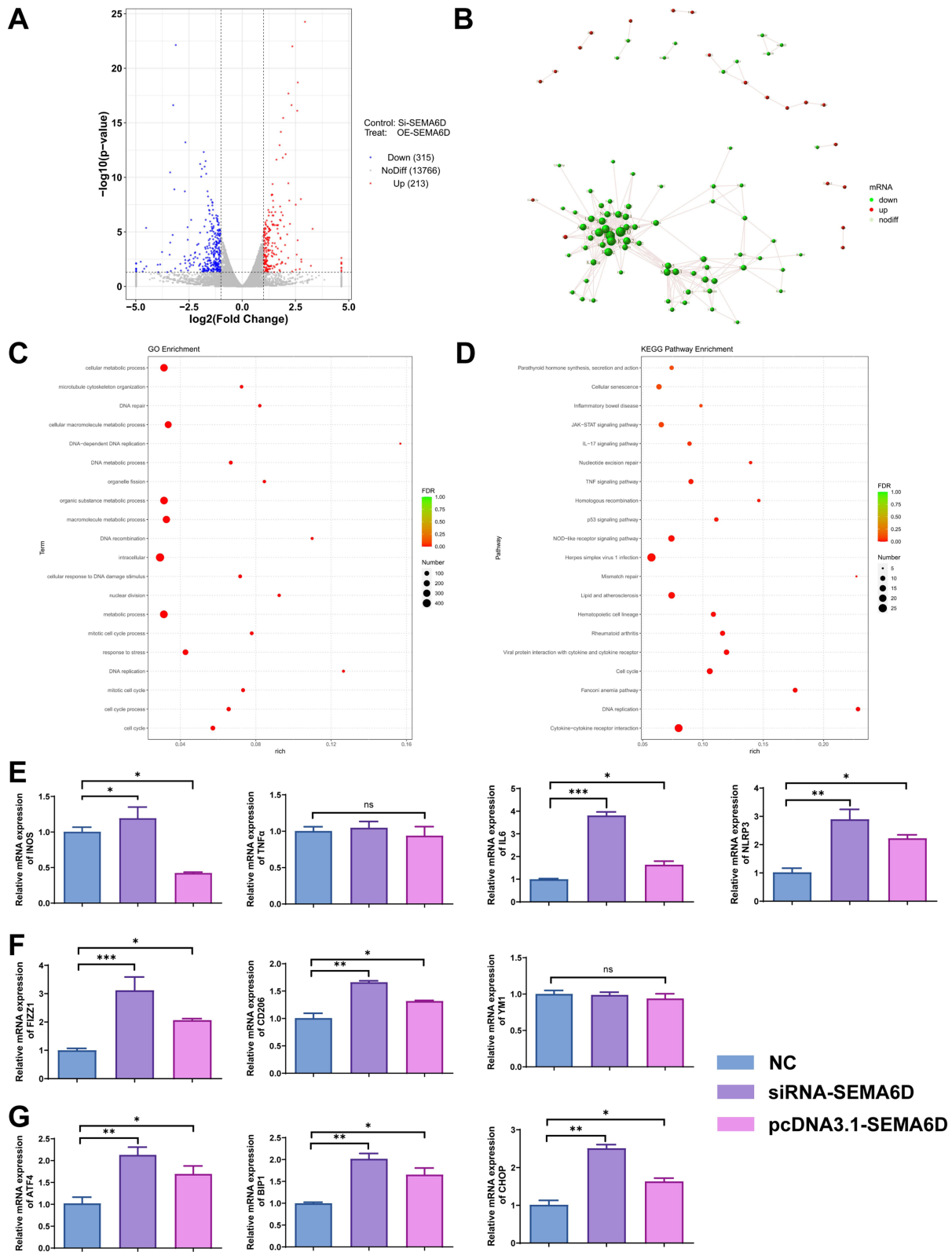


Figure 1 The upregulation of SEMA6D inhibits the polarization of M1 macrophages and downregulates the expression of endoplasmic reticulum stress-related genes. **(A)** Volcano plot of differentially expressed genes (DEGs) between siRNA-SEMA6D and pcDNA3.1-SEMA6D. **(B)** Protein interaction analysis. **(C)** Gene Ontology (GO) enrichment analysis. **(D)** The Kyoto encyclopedia of genes and genomes (KEGG) analysis of RAW264.7. **(E)** The M1 macrophage relative mRNA levels in RAW264.7 cells. **(F)** The M2 macrophage relative mRNA levels in RAW264.7 cells. **(G)** The endoplasmic reticulum stress relative mRNA levels in RAW264.7 cells. *p < 0.05; **p < 0.01; ***p < 0.001; ns = no significance. All data are shown as mean ± SD.

than two-fold, although no significant differences were observed for TNF α (Figure 1E). Additionally, NLRP3 expression was higher in the siRNA-SEMA6D group compared to the pcDNA3.1-SEMA6D group. M2 markers revealed that SEMA6D did not promote M2 macrophage polarization (Figure 1F). Moreover, genes associated with endoplasmic reticulum stress were significantly upregulated in the siRNA-SEMA6D group (Figure 1G). In summary, upregulation of SEMA6D may inhibit M1 macrophage polarization by reducing endoplasmic reticulum stress and NLRP3 expression.

The Upregulation of SEMA6D Inhibits the Polarization of M1 Macrophages

To further investigate the effect of SEMA6D on macrophage polarization, RAW264.7 cells were pretreated with LPS to induce an inflammatory phenotype. As shown in Figure 2A, treatment with pcDNA3.1-SEMA6D resulted in a significant reduction in iNOS expression. Quantitative fluorescence analysis revealed that the expression level of iNOS in the pcDNA3.1-SEMA6D group was only half of that observed in the siRNA-SEMA6D group (Figure 2B). Additionally, the supernatant collected from the culture medium after centrifugation was analyzed using ELISA to measure IL-1 β and IL-6 levels (Figure 2C). The results were consistent with the immunofluorescence findings, showing that upregulation of SEMA6D reduced the secretion of inflammatory cytokines by macrophages. Subsequently, qRT-PCR was performed to examine the expression levels of related genes. As shown in Figure 2D, upregulation of SEMA6D suppressed the expression of IL-1 β , iNOS, and IL-6 genes. To further investigate differences at the protein level, Western blot analysis was conducted (Figure 2E and F). The results demonstrated that increased SEMA6D expression was associated with reduced levels of iNOS, IL-6, and IL-1 β . Overall, these findings suggest that SEMA6D expression can inhibit the M1 polarization of macrophages.

SEMA6D Inhibits M1 Polarization and Reduces the Release of Pro-Inflammatory Mediators by Suppressing the Expression of NLRP3

Next, we sought to elucidate the underlying mechanism by which SEMA6D inhibits macrophage polarization. Previous studies have reported that the activation of NLRP3 promotes the differentiation of macrophages into an inflammatory phenotype and enhances the secretion of inflammatory factors such as IL-1 β .^{28–30} To investigate this, we examined the expression of NLRP3 in different groups via immunofluorescence. The results showed that inhibition of SEMA6D promoted the expression of NLRP3 (Figure 3A and B). Subsequently, flow cytometry was employed to analyze the expression of the M1 macrophage marker CD86 across different groups. Proportion of M1 macrophages in the siRNA-SEMA6D group was 31.4%, which was higher than that in the pcDNA3.1-SEMA6D group (29.3%) and the control group (30.5%) (Figure 3C and D). These findings suggest that SEMA6D may reduce M1 polarization of macrophages under inflammatory conditions by downregulating NLRP3 expression. Furthermore, Western blot analysis was performed to assess the protein levels of caspase-1 and NLRP3. The results confirmed that SEMA6D inhibits the expression of NLRP3 (Figure 3E and F). In conclusion, SEMA6D suppresses the polarization of macrophages toward the M1 phenotype by downregulating NLRP3 expression.

The Upregulation of SEMA6D Inhibits the Expression of ASC in Macrophages and Reduces the Release of Pro-Inflammatory Mediators

ASC is closely associated with the assembly and activation of the NLRP3 inflammasome,^{31–33} 为 Therefore, we further explored the role of SEMA6D in ASC-mediated NLRP3 activation. As shown in Figure 4A, immunofluorescence analysis revealed that the expression of ASC in the pcDNA3.1-SEMA6D group was lower than that in the other groups. ATP, used as an activator of NLRP3 here,^{34,35} significantly increased NLRP3 expression following treatment. However, when both pcDNA3.1-SEMA6D and ATP were applied, we observed that the upregulation of SEMA6D inhibited the activation of NLRP3 induced by ATP (Figure 4B). This suggests that SEMA6D is closely associated with ASC expression and can downregulate ASC levels. Flow cytometry results further demonstrated that the upregulation of SEMA6D reduced ASC-induced M1 polarization of macrophages (Figure 4C and D). Consistently, PCR results confirmed that SEMA6D expression led to the downregulation of M1 macrophage marker genes (Figure 4E–G). Finally, Western blot analysis was performed to

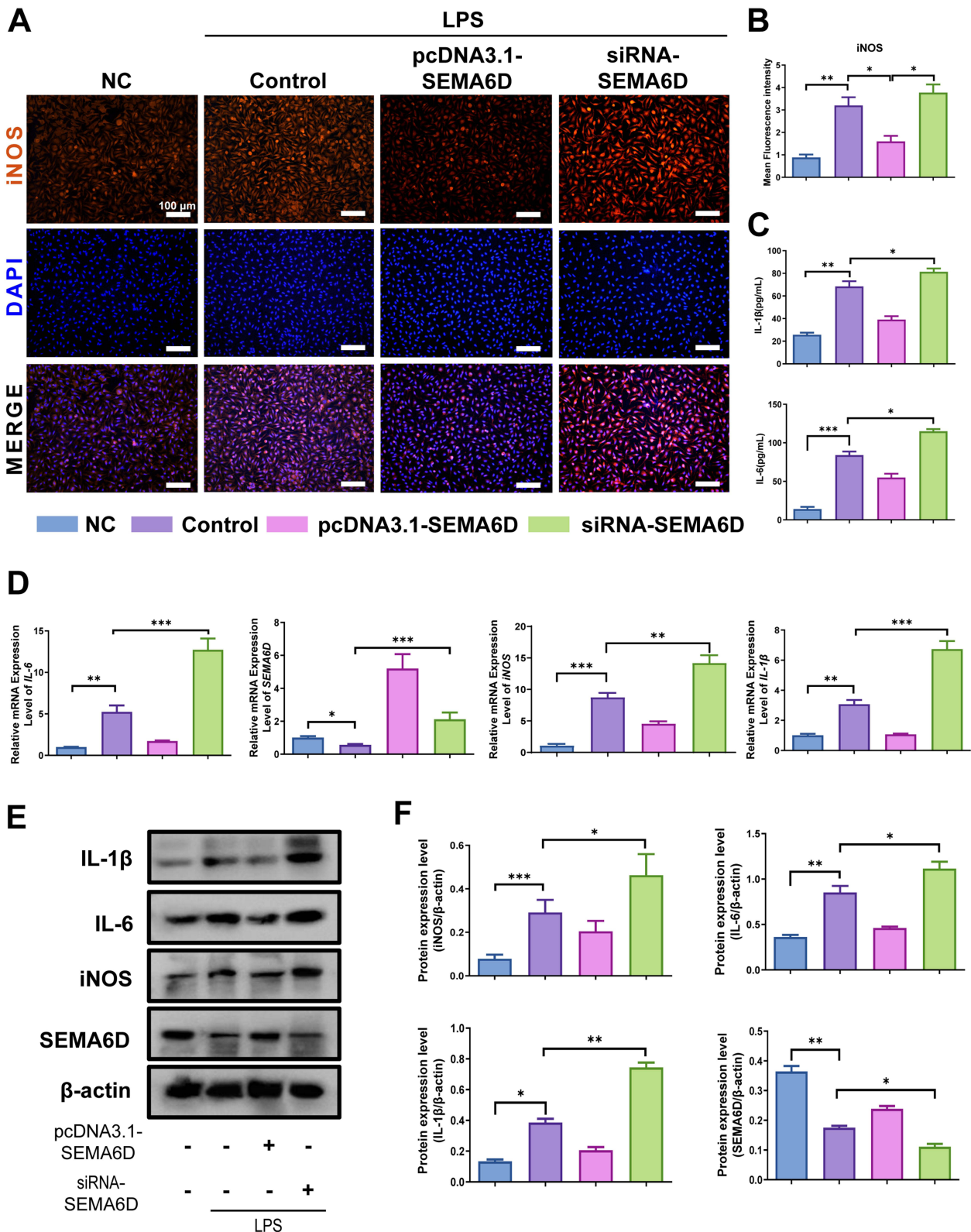


Figure 2 The upregulation of SEMA6D inhibits the polarization of M1 macrophages. **(A)** Immunofluorescence analysis of iNOS in the cells after 24 h. **(B)** Quantitative analysis of iNOS immunofluorescence (n=3). **(C)** Concentration of IL-6 and IL-1β in the supernatant of the culture medium after 100 ng/mL LPS treatment with siRNA-SEMA6D or pcDNA3.1-SEMA6D for 24 h. (n=3). **(D)** The mRNA levels in cells after treatment with PBS, LPS, LPS + pcDNA3.1-SEMA6D, LPS + siRNA-SEMA6D. **(E)** Protein in levels of IL-1β, IL-6, iNOS, SEMA6D in the cells after 100 ng/mL LPS treatment with siRNA-SEMA6D or pcDNA3.1-SEMA6D for 24 h. **(F)** Quantitation of Western blotting; n = 3. *p < 0.05; **p < 0.01; ***p < 0.001; ns = no significance. All data are shown as mean ± SD.

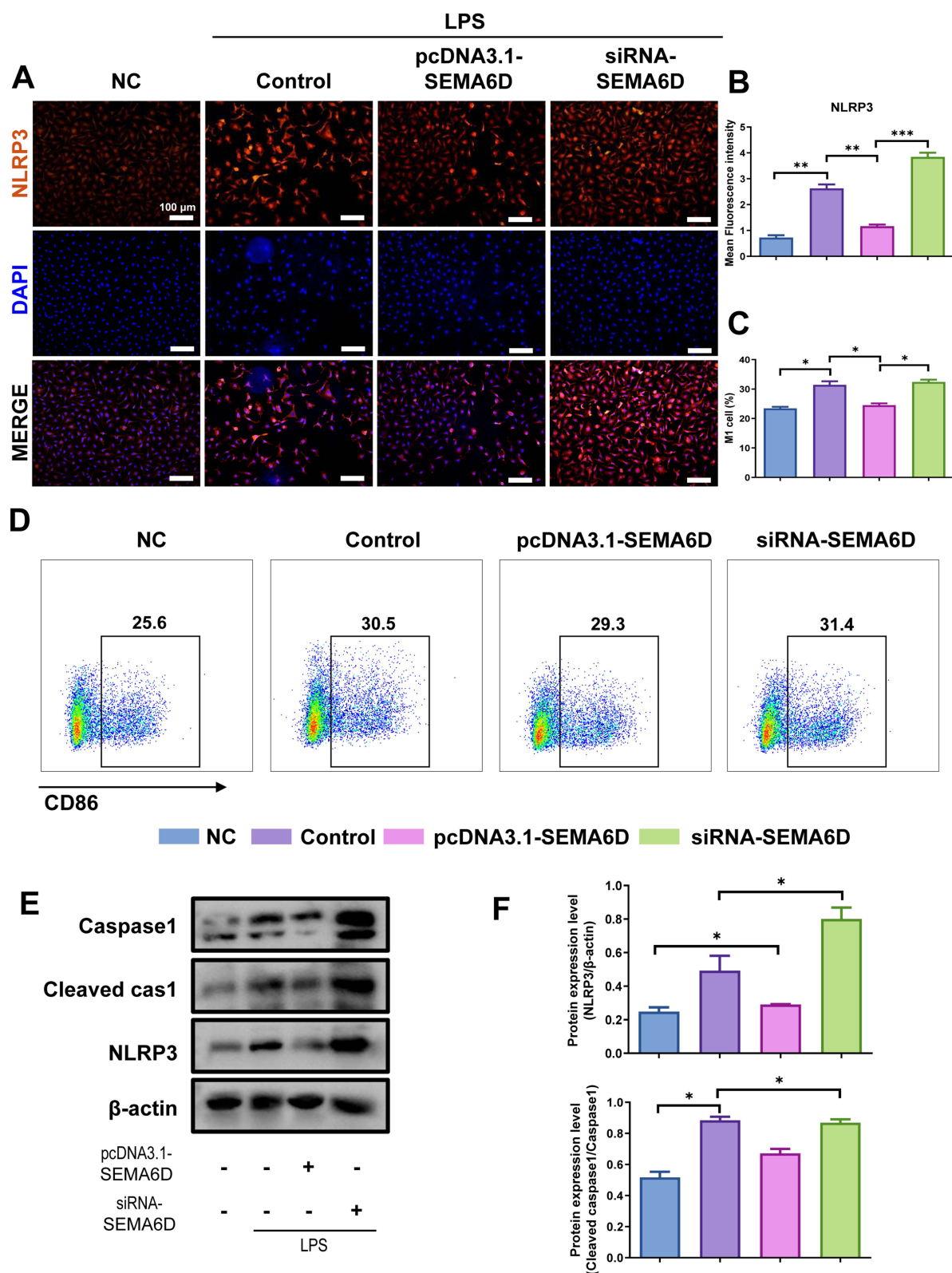


Figure 3 SEMA6D inhibits M1 polarization and reduces the release of pro-inflammatory mediators by suppressing the expression of NLRP3. **(A)** Immunofluorescence analysis of NLRP3 in the cells after 24 h. **(B)** Quantitative analysis of NLRP3 immunofluorescence (n=3). **(C)** Flow cytometric quantitative analysis of CD86 positive cells percentage (n=3). **(D)** Flow cytometric analysis of CD86 expression. **(E)** Protein in levels of Caspase-1, Cleaved caspase-1, NLRP3 in the cells after 100 ng/mL LPS treatment with siRNA-SEMA6D or pcDNA3.1-SEMA6D for 24 h. **(F)** Quantitation of Western blotting; n = 3. * p < 0.05; ** p < 0.01; *** p < 0.001; ns = no significance. All data are shown as mean \pm SD.

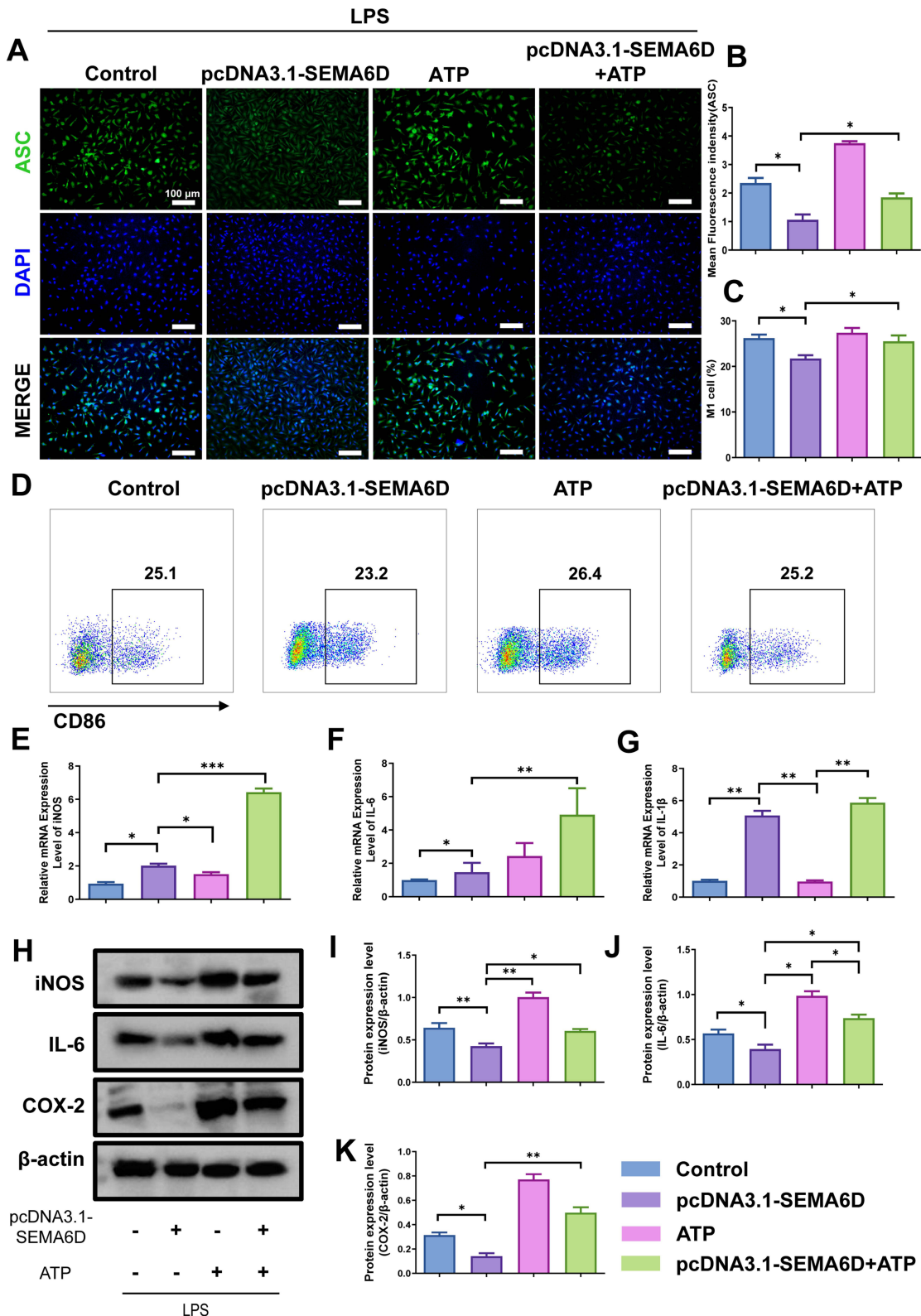


Figure 4 The upregulation of SEMA6D inhibits the expression of ASC in macrophages and reduces the release of pro-inflammatory mediators. **(A)** Immunofluorescence analysis of ASC in the cells after 24 h. **(B)** Quantitative analysis of ASC immunofluorescence (n=3). **(C)** Flow cytometric quantitative analysis of CD86 positive cells percentage (n=3). **(D)** Flow cytometric analysis of CD86 expression. The mRNA levels in cells after treatment with LPS, LPS + pcDNA3.1-SEMA6D, 5 μ mol/L ATP, LPS + pcDNA3.1-SEMA6D + 5 nmol/L ATP for 24 h, **(E)** iNOS **(F)** IL-6 **(G)** IL-1 β . **(H)** Protein in levels of iNOS, IL-6, COX-2 in the cells after 100 ng/mL LPS treatment with 5 nmol/L ATP or pcDNA3.1-SEMA6D for 24h. Quantitation of Western blotting **(I)** iNOS, **(J)** IL-6, **(K)** COX-2; n = 3. * p < 0.05; ** p < 0.01; *** p < 0.001; ns = no significance. All data are shown as mean \pm SD.

validate the protein levels of M1 macrophage markers (Figure 4H). The results showed that the levels of iNOS, IL-6, and COX-2 exhibited a negative correlation with SEMA6D expression (Figure 4I–K).

The Expression of SEMA6D Can Reduce Endoplasmic Reticulum Stress in Macrophages Under Inflammatory Conditions

Endoplasmic reticulum (ER) stress has been shown to have a strong association with macrophage polarization during inflammatory responses.^{36–38} Therefore, we sought to investigate the role of SEMA6D in regulating ER stress in macrophages. Thapsigargin (Th), a widely used ER stress activator, was employed in this study.^{39,40} As shown in Figure 5A and B, treatment of RAW264.7 cells with Th significantly upregulated the expression of ER stress markers, including ATF4, BIP-1, CHOP, and IRE1 α , compared to the NC group. Next, we treated cells with a combination of Th and pcDNA3.1-SEMA6D. Western blot analysis revealed that the expression of ER stress-related proteins was markedly increased in the LPS+Th group. However, in the LPS+Th+pcDNA3.1-SEMA6D group, upregulation of SEMA6D notably reduced the protein levels of these markers. Interestingly, in the LPS+pcDNA3.1-SEMA6D group, the addition of LPS did not counteract the downregulation of ER stress marker proteins induced by pcDNA3.1-SEMA6D. Remarkably, the expression levels of these proteins in this group were even lower than those in the NC group, indicating that under inflammatory conditions, the upregulation of SEMA6D significantly suppresses ER stress (Figure 5C and D). Transmission electron microscopy (TEM) images further demonstrated the inhibitory effect of SEMA6D on ER stress (Figure 5E). In the Th+pcDNA3.1-SEMA6D group, no signs of ER enlargement or vacuolization, which were observed in the Th-treated group, were detected. The ER morphology in the Th+pcDNA3.1-SEMA6D group closely resembled that of the NC group.

The Expression of SEMA6D Delays the Progression of Osteoarthritis

Cartilage and synovium from OA rats were collected for histological evaluation. H&E staining revealed severe tissue damage in the OA group, whereas the pcDNA3.1-SEMA6D group exhibited less pronounced tissue defects compared to the OA group. Safranin O-Fast Green and Masson's trichrome staining demonstrated that the OA group experienced significant cartilage structural degradation, severe collagen breakdown, and tide line displacement. In contrast, cartilage destruction was markedly alleviated in the AAV6-SEMA6D group compared to the OA group. Notably, the AAV6-SEMA6D group exhibited reduced inflammatory infiltration in synovial tissues compared to the OA group (Figure 6A). To further evaluate the inflammation levels in synovial tissue (Figure 6B and C), immunohistochemistry results showed that the number of IL-6 and iNOS-positive cells in the AAV6-SEMA6D group was lower than that in the OA group. Additionally, the expression level of NLRP3 in the AAV6-SEMA6D group was also reduced compared to the OA group, consistent with the *in vitro* findings. These results indicate that SEMA6D lowers the expression of inflammatory factors by inhibiting the NLRP3 inflammasome in macrophages. Subsequently, we assessed the distribution of M1 macrophages in synovial tissue using immunofluorescence (Figure 6D and E). The results showed reduced M1 macrophage infiltration in synovial tissues from the AAV6-SEMA6D group compared to the OA group, with a negative correlation between SEMA6D expression and M1 macrophage marker levels. These findings suggest that SEMA6D alleviates synovial inflammation by suppressing M1 macrophage polarization, thereby mitigating the progression of OA.

Discussion

OA is a common disabling disease. To date, the exact pathogenesis of OA remains unclear. However, with the ongoing advancements in OA research, abnormalities in immune cells have been identified as key mediators in the development of OA. Innate immunity often serves as the first line of defense activated upon tissue injury. Although the involvement of adaptive immunity, particularly T cell-mediated responses, in synovitis has been widely demonstrated in both OA and rheumatoid arthritis (RA),^{39–41} innate immunity is the initial factor in the cascade of events related to synovitis in OA. Unlike adaptive immunity, innate responses rely on the recognition of pathogen-associated molecular patterns (PAMPs), activated by components derived from pathogens, or danger-associated molecular patterns (DAMPs), activated by cellular contents released from necrotic cells in sites of tissue damage.⁴² DAMPs trigger an inflammatory response by

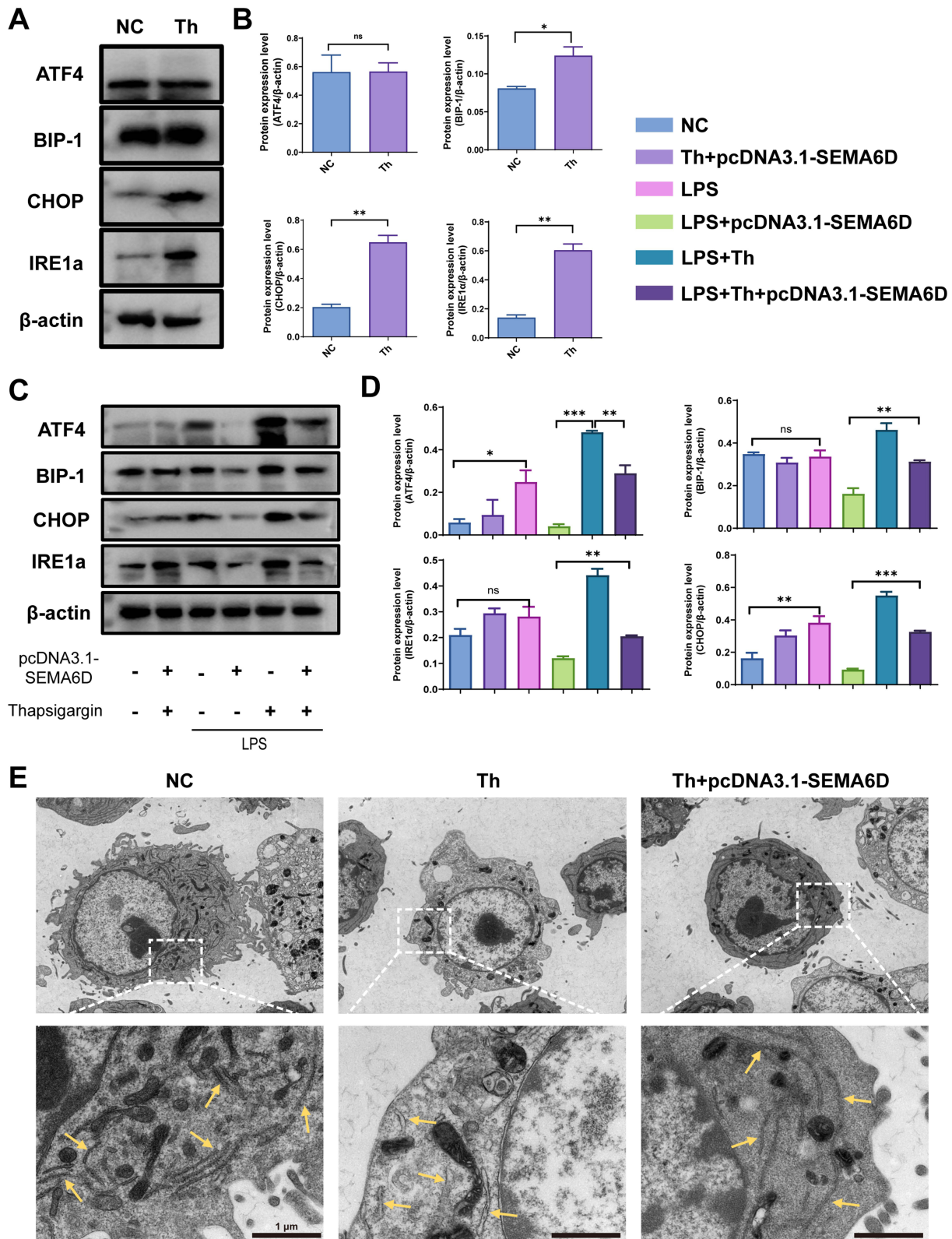


Figure 5 The expression of SEMA6D can reduce endoplasmic reticulum stress in macrophages under inflammatory conditions. **(A)** Protein in levels of ATF4, BIP-1, CHOP and IRE1α in the cells treatment with 1 μmol/L thapsigargin for 24 h. **(B)** Quantitation of Western blotting; n = 3. **(C)** Protein in levels of ATF4, BIP-1, CHOP and IRE1α in the cells after 100 ng/mL LPS treatment with 1 μmol/L thapsigargin or pcDNA3.1-SEMA6D for 24 h. **(D)** Quantitation of Western blotting; n = 3. **(E)** Representative TEM images of macrophages treated with thapsigargin and pcDNA3.1-SEMA6D. The yellow arrow indicates the endoplasmic reticulum. *p < 0.05; **p < 0.01; ***p < 0.001; ns = no significance. All data are shown as mean ± SD.

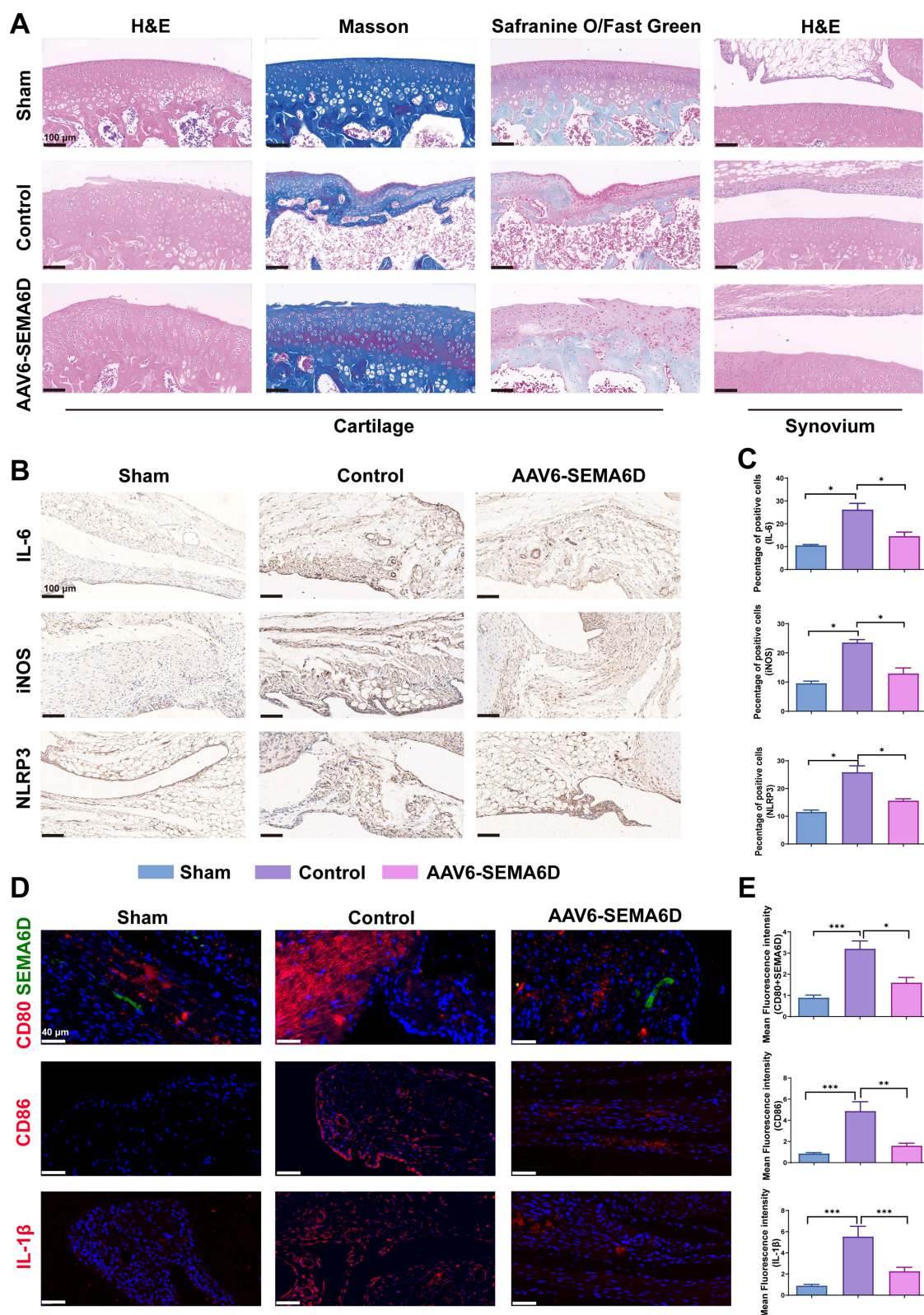


Figure 6 The expression of SEMA6D delays the progression of osteoarthritis. **(A)** Safranin O/Fast Green, H&E and Masson staining of knee joints from Sham, Control, AAV6-SEMA6D. **(B)** Representative microscopic photographs of IL-6, iNOS and NLRP3 immunohistochemical staining. **(C)** Quantitative analysis of IL-6, iNOS and NLRP3 expression (n=3). **(D)** Immunofluorescence staining of CD80, SEMA6D, CD86, IL-1 β in the synovium. **(E)** Quantitative analysis of CD80, SEMA6D, CD86, IL-1 β immunofluorescence (n=3). *p < 0.05; **p < 0.01; ***p < 0.001. All data are shown as mean \pm SD.

interacting with receptors on innate immune cells, known as pattern recognition receptors (PRRs). Once activated, innate immunity is expected to initiate a response promoting tissue regeneration or repair. Thus, during OA progression, innate immune cells are the first immune cells to be activated. However, abnormal cell-mediated activation of immune cells can trigger an antagonistic immune response that exacerbates tissue damage. Immune cells, which normally help maintain tissue homeostasis, become contributors to the destruction of normal tissue. Studies have revealed significant differences in immune cell proportions between normal and osteoarthritic tissues.⁴³ In OA synovial tissues, plasma cells, M1 and M2 macrophages, activated dendritic cells (DCs), and resting mast cells (MCs) are significantly increased. This suggests that the imbalance in synovial immune homeostasis is a critical factor in OA progression. Liu et al reported that reduced autophagy promotes the production of mito-ROS, which further enhances inflammasome activation and subsequent IL-1 β processing.⁴⁴ Additionally, exosomes derived from inflammatory fibroblast-like synoviocytes exacerbate OA by promoting glycolysis in macrophages;⁴⁵ Sun et al demonstrated that SHP 099 slows OA progression by inhibiting M1 macrophage polarization.⁴⁶ These findings indicate that the homeostasis of synovial macrophages is closely related to OA progression, highlighting synovial macrophages as potential therapeutic targets for OA.

Our previous research reported that SEMA6D is negatively regulated by miR-7 and promotes the catabolic and anabolic activity of C28/I2 chondrocytes through the p38 signaling pathway.²⁵ We revealed that SEMA6D is a direct target gene of miR-7, exhibiting a negative regulatory relationship in both in vitro and in vivo models. Animal experiments confirmed that SEMA6D can alleviate OA progression in rats and promote cartilage matrix deposition. Considering that OA progression is not solely driven by a single cell type or tissue, we became particularly interested in the effect of SEMA6D on synovial macrophages in OA. Notably, the regulation of synovial macrophages by SEMA6D has not been previously reported. This study may hold potential value for the development of OA therapies.

To further investigate the molecular mechanisms underlying SEMA6D function, we treated macrophages with siRNA-SEMA6D and pcDNA3.1-SEMA6D, respectively, and conducted transcriptomic sequencing. Analysis revealed that the NOD-like receptor signaling pathway and the TNF signaling pathway were upregulated in RAW264.7 cells treated with siRNA-SEMA6D. To validate these findings, we examined the expression of M1 and M2 macrophage markers via qRT-PCR. The results showed that M1 macrophage markers were significantly upregulated following siRNA-SEMA6D transfection, further supporting the activation of inflammatory pathways. Additionally, we observed upregulation of NLRP3 and genes associated with ER stress.

Next, we evaluated the phenotypic changes in macrophages under LPS stimulation following transfection with si-SEMA6D or pcDNA3.1-SEMA6D to clarify the function of SEMA6D. The results revealed that upregulation of SEMA6D inhibited macrophage polarization toward the pro-inflammatory M1 phenotype. Subsequently, we explored SEMA6D's regulatory pathways and identified NLRP3 and ASC as its downstream targets. Overexpression of SEMA6D via pcDNA3.1-SEMA6D significantly downregulated the expression of both NLRP3 and ASC. These findings suggest that SEMA6D suppresses M1 macrophage polarization by inhibiting NLRP3 inflammasome activation. Moreover, we confirmed that SEMA6D effectively reduces ER stress in macrophages under inflammatory conditions, providing further insight into the mechanisms by which SEMA6D regulates macrophage phenotypes. Finally, in a rat OA model, we validated the in vitro findings by demonstrating that SEMA6D upregulation reduced the infiltration of pro-inflammatory M1 macrophages into synovial tissues, diminished synovial inflammation, and consequently relieve OA progression.

In conclusion, the above findings demonstrate that SEMA6D acts as an inhibitory factor in OA by mediating synovial inflammation through the regulation of macrophage phenotypes, thereby mitigating OA development. Despite these promising results, there are still limitations to our work. The knockout and multi-sex animal experiments will provide more convincing results for the experimental outcomes. In addition, appropriately including human samples will provide practical significance to the research results. These tasks need to be given attention in the subsequent research in order to fully determine the potential of these biomarkers in improving the management and treatment outcomes of patients with osteoarthritis.

Conclusions

In this study, we identified SEMA6D as a critical regulator of macrophage polarization. SEMA6D inhibits the activation of the NLRP3 inflammasome by ASC, thereby blocking the release of downstream pro-inflammatory mediators such as

IL-1 β . Furthermore, the upregulation of SEMA6D reduces endoplasmic reticulum stress and alleviates inflammation associated with it. In a rat OA model, SEMA6D upregulation decreased M1 macrophage infiltration in synovial tissues and downregulated inflammatory factor levels, thereby mitigating OA progression.

Data Sharing Statement

The data supporting the results of this study can be provided upon the reasonable request of the first corresponding author.

Funding

This study was supported by the Qinghai Province Basic Research Project (2024-ZJ-760), the Changzhou Sci&Tech Program (CZ20240029), the Science and Technology Project of Changzhou Health Commission (ZD202319 and ZD202339), the Basic Research Project of Changzhou Medical Center of Nanjing Medical University (CMCB202314), and the Top Talent of Changzhou “The 14th Five-Year Plan” High-Level Health Talents Training Project (2022CZBJ059 and 2022CZBJ061).

Disclosure

The authors declare no conflict of interest.

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