

Sustained-release aliphatic acid-loaded hydrogel attenuates intervertebral disc degeneration through regulation of the MIF-SERPINE1-PI3K-Akt/NF- κ B axis

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Abstract

Intervertebral disc degeneration (IVDD) is the leading cause of global chronic low back pain, primarily driven by sustained inflammatory microenvironment activation and progressive extracellular matrix (ECM) degradation. Current IVDD therapeutic strategies are limited by unclear core pathogenic mechanisms and rapid in vivo clearance of conventional small-molecule drugs, failing to achieve persistent anti-inflammatory and anti-degenerative effects. Macrophage migration inhibitory factor (MIF) acts as a pivotal pro-inflammatory mediator in musculoskeletal degenerative disorders, yet its exact downstream effector network and targeted therapeutic intervention strategies in IVDD remain poorly defined. To address these limitations, we systematically explored the MIF-centered pathological mechanism and developed an innovative sustained-release hydrogel delivery system for precise IVDD treatment. In this study, quantitative proteomics of clinical degenerative nucleus pulposus (NP) specimens with different Pfirrmann grades was performed to screen key pathogenic molecules. Clinical specimen validation and rat IVDD model experiments confirmed that MIF was highly upregulated in severe degenerative NP tissues. Mechanistically, MIF directly binds to SERPINE1 with high affinity ($K_d = 12.8 \text{ nM}$), which further activates the PI3K-Akt/NF- κ B pro-inflammatory signaling cascade and ultimately promotes the secretion of TNF- α and IL-1 β . Through immobilized MIF affinity chromatography screening from *Eucommia ulmoides*, alphitolic acid was identified as a high-affinity MIF antagonist with a K_d value of $1.85 \mu\text{M}$. To improve the local retention and bioavailability of alphitolic acid, we fabricated an injectable hyaluronic acid-dopamine composite hydrogel (Alp@HDgel) with favorable biocompatibility, stable porous microstructure and mechanical properties, which achieved more than 60% cumulative sustained drug release within 48 hours. In vitro mechanistic assays including Co-IP, SPR and ITC verified the targeted inhibitory effect of alphitolic acid on the MIF-SERPINE1 axis, and in vivo rat puncture-induced IVDD model experiments demonstrated that Alp@HDgel effectively suppressed local inflammatory responses, restored ECM homeostasis, and rescued disc structural deterioration, exhibiting significantly superior therapeutic efficacy compared with free alphitolic acid and blank hydrogel. In conclusion, this study defines the critical MIF-SERPINE1-PI3K-Akt/NF- κ B regulatory axis that mediates IVDD inflammatory degeneration. The engineered sustained-release alphitolic acid hydrogel achieves targeted blockade of MIF-driven pathological cascades and effectively reverses IVDD progression, providing a feasible and disease-modifying therapeutic strategy for clinical IVDD treatment.

Highlights

- Macrophage migration inhibitory factor (MIF) is a critical driver of intervertebral disc degeneration (IVDD)
- MIF directly binds SERPINE1 and activates the PI3K-Akt/NF- κ B pro-inflammatory signaling cascade
- Alphitolic acid, isolated from *Eucommia ulmoides*, acts as a high-affinity MIF antagonist ($K_d = 1.85 \mu\text{M}$)
- Alp@HDgel reverses IVDD in rats by restoring ECM homeostasis and suppressing inflammation

1. Introduction

Low back pain (LBP) is the leading cause of years lived with disability worldwide, affecting approximately 25% of the adult population at any given time and exacting a staggering \$100 billion annual economic toll on the U.S. healthcare system alone[1–3]. Intervertebral disc degeneration (IVDD) is the primary pathological driver of LBP, characterized by progressive extracellular matrix (ECM) degradation, chronic low-grade inflammation, and irreversible loss of nucleus pulposus (NP) cellularity[4]. Despite decades of intensive research, clinical management of IVDD remains exclusively palliative, focusing on symptomatic pain relief and surgical intervention for end-stage disease. These approaches fail to address the underlying degenerative mechanisms and are associated with high recurrence rates and significant perioperative complications. Therefore, there is an urgent unmet clinical need to identify actionable pathogenic targets and develop disease-modifying therapies that can halt or reverse IVDD progression.

Traditional Chinese herbs (TCHs) have emerged as a rich source of novel therapeutics for degenerative diseases, due to their multi-targeted regulatory effects and favorable safety profiles. *Eucommia ulmoides* Oliv., a classic TCH widely used for musculoskeletal disorders for over 2000 years, has been shown to exhibit potent anti-inflammatory, antioxidant, and bone-protective activities in preclinical studies[5, 6]. However, the specific bioactive components of *E. ulmoides* responsible for its therapeutic effects in IVDD remain poorly characterized, and their molecular targets have not been definitively identified. This critical knowledge gap has hindered the clinical translation of *E. ulmoides*-based therapies for IVDD and limited their acceptance in Western medicine.

Macrophage migration inhibitory factor (MIF) is a pleiotropic pro-inflammatory cytokine that plays a central role in tissue inflammation and degeneration. Elevated MIF expression has been reported in various degenerative musculoskeletal disorders, including osteoarthritis and rheumatoid arthritis, where it drives inflammatory cytokine production and ECM breakdown[7–10]. While preliminary studies have suggested a potential role for MIF in IVDD, its precise molecular mechanisms and downstream effectors remain completely unelucidated. The serine protease inhibitor (SERPIN) superfamily regulates ECM remodeling and inflammatory responses, and several members, including SERPINE1, are upregulated in degenerative disc tissue[11]. However, no studies to date have investigated the functional interaction between MIF and SERPIN family members in IVDD pathogenesis.

A major unaddressed challenge in IVDD therapy is the avascular nature of the intervertebral disc, which limits the efficacy of systemic drug delivery by preventing sufficient drug accumulation in the disc tissue[12]. Injectable hydrogels have emerged as an ideal platform for local drug delivery in IVDD, offering excellent biocompatibility, shear-thinning properties, and sustained drug release capabilities[13]. Hyaluronic acid (HA), a major component of the disc ECM, and dopamine (DA), a bioadhesive molecule, are particularly attractive materials for hydrogel fabrication, as they provide a supportive microenvironment for NP cell survival and ECM repair[14, 15].

In this study, we employed quantitative proteomics to identify Complement and coagulation cascades pathway (including SERPIND1, SERPINE1, and SERPINF2) as a critical pro-degenerative target in IVDD. We then elucidated the molecular mechanism by which MIF drives IVDD progression via the SERPINE1-mediated PI3K-Akt/NF- κ B pathway. Using immobilized MIF chromatography, we screened aliphatic acid from *E. ulmoides* as a high-affinity MIF antagonist. Finally, we developed an injectable HA-DA hydrogel for sustained local delivery of aliphatic acid and demonstrated its superior therapeutic efficacy in a rat IVDD model. Our findings provide a novel disease-modifying strategy for IVDD and highlight the potential of natural product-based hydrogel therapies for degenerative musculoskeletal disorders.

2. Materials and methods

2.1 Clinical samples

Clinical nucleus pulposus (NP) tissues were obtained from 22 patients diagnosed with IVDD who underwent surgical treatment (microdiscectomy or lumbar fusion) at Tangdu Hospital between January 2022 and December 2023. Patients were classified into Pfirrmann grade II ($n = 10$) and grade IV ($n = 12$) based on preoperative magnetic resonance imaging (MRI) findings. All patients provided written informed consent, and the study was approved by the Ethics Committee of Tangdu Hospital (Approval No. 202102-16) and followed the guidelines of the Declaration of Helsinki. NP cells (NPCs) were isolated from NP tissues as previously described[16]. Briefly, NP tissues were minced into small pieces (1–2 mm³), digested with 0.25% trypsin (Gibco, USA) for 30 min at 37°C, followed by digestion with 0.1% collagenase type II (Sigma-Aldrich, USA) for 4 h at 37°C. The digested cells were filtered through a 70 μ m cell strainer, centrifuged at 1000 rpm for 5 min, and resuspended in DMEM/F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). Cells were cultured in a humidified incubator at 37°C with 5% CO₂, and the medium was changed every 2–3 days. Passages 3–5 NPCs were used for all subsequent experiments.

2.2 Animal models

Seven-week-old male Sprague-Dawley (SD) rats (200–250 g) were purchased from the Experimental Animal Center of Air Force Medical University (Xi'an, China) (License No. SCXK 2021-0008). All rats were housed in a specific pathogen-free (SPF) environment with a 12 h light/dark cycle, constant temperature (22 $\pm$ 2°C), and constant humidity (50 $\pm$ 5%), with free access to food and water. All surgical interventions, treatments and postoperative animal care procedures were performed in strict accordance with the requirements of the Animal Care and Use Committee of Air Force Medical University (202203-08) and the NIH Guide for the Care and Use of Laboratory Animals, and adhered to the ARRIVE guidelines. The rat IVDD model was established by percutaneous puncture as previously described[17]. Briefly, rats were anesthetized with 10% chloral hydrate (3 mL/kg, intraperitoneal injection). After shaving and disinfection of the dorsal skin, the L5-L6 intervertebral disc was localized by palpation. A 21-gauge needle was inserted perpendicular to the skin, punctured through the annulus fibrosus into the nucleus pulposus, and retained for 30 s before withdrawal. The control group received only skin incision without needle

puncture. One week after model establishment, rats were randomly divided into 4 groups (n = 6 per group): IVDD group (no treatment), HDgel group (intradiscal injection of blank hydrogel), Alp group (intradiscal injection of free aliphatic acid), and Alp@HDgel group (intradiscal injection of aliphatic acid-loaded hydrogel). The injection volume was 10 μ L per disc. Rats were sacrificed at 4 and 8 weeks post-treatment, and L5-L6 intervertebral discs were collected for subsequent experiments.

2.3 Surface Plasmon Resonance (SPR) Analysis

SPR analysis was performed using a Biacore T200 instrument (Cytiva, USA) at 25°C. MIF protein was immobilized on a CM5 sensor chip using the amine coupling method. SERPIND1, SERPINE1, SERPINF2, aliphatic acid, and 3-indoleacrylic acid were diluted in running buffer (PBS, pH 7.4) to different concentrations and injected into the flow cell at a flow rate of 30 μ L/min. The association time was 180 s, and the dissociation time was 300 s. The sensor chip was regenerated with 10 mM glycine-HCl (pH 2.5) for 30 s. The binding curves were fitted using Biacore T200 Evaluation Software, and the equilibrium dissociation constant (K_d) was calculated.

2.4 Isothermal Titration Calorimetry (ITC) Analysis

ITC analysis was performed using a MicroCal PEAQ-ITC instrument (Malvern Panalytical, UK) at 25°C. MIF protein was dissolved in PBS (pH 7.4) to a concentration of 10 μ M, and aliphatic acid and 3-indoleacrylic acid were dissolved in the same buffer to a concentration of 100 μ M. The ligand solution was injected into the sample cell containing MIF protein with 19 injections (first injection: 0.4 μ L, subsequent injections: 2 μ L each) at intervals of 120 s. The stirring speed was 750 rpm. The data were analyzed using MicroCal PEAQ-ITC Analysis Software to obtain the thermodynamic parameters, including K_d , ΔH , ΔS , and ΔG .

2.5 Preparation and Characterization of Immobilized MIF Column

Tag9-MIF was expressed in *Escherichia coli* (E. coli) BL21 (DE3) cells and purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. The purity of Tag9-MIF was verified by SDS-PAGE and WB. Streptavidin-modified silica gels were purchased from Sigma-Aldrich (USA). MIF was immobilized on the silica gels via the high-affinity interaction between Tag9 and streptavidin. The immobilization efficiency was verified by X-ray photoelectron spectroscopy (XPS) analysis. The XPS survey spectra of naked silica gels and MIF-coated silica gels were recorded using an XPS spectrometer (Thermo Fisher Scientific, USA), and the atomic percentages of elements on the surface were analyzed.

2.6 Screening of MIF-Specific Ligands from *Eucommia ulmoides*

Eucommia ulmoides bark was purchased from a local Chinese medicine store (Xi'an, China) and authenticated by Professor Bo Liao (Tangdu Hospital, Air Force Medical University). The bark was dried, crushed, and extracted with 70% ethanol under reflux for 2 h. The extract was filtered, concentrated

under reduced pressure, and lyophilized to obtain the crude extract. The crude extract was dissolved in methanol and filtered through a 0.22 μm filter membrane. The MIF-immobilized column was equilibrated with methanol, and the crude extract was loaded onto the column. The column was washed with methanol to remove non-specifically bound components, and the specifically bound components were eluted with methanol containing 0.1% formic acid. The eluted components were analyzed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) to identify potential MIF-specific ligands. Known MIF ligands (MIF098, ISO-1, BTZO-1) were used as positive controls to verify the specificity of the screening system.

2.7 Statistical Analysis

All experiments were performed in triplicate ($n = 3-5$). Data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, USA). Differences between two groups were analyzed using unpaired two-tailed Student's t-test. Differences among multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.

2.8 Others

Additional experimental procedures and characterization methods are provided in the Supplementary Materials.docx.

3. Results

3.1 MIF is significantly overexpressed in IVDD NPCs

In clinical samples, qRT-PCR and WB results showed that MIF mRNA and protein levels were significantly higher in NPCs from Pfirrmann grade IV IVDD patients than in those from grade II patients ($n = 5$, $P < 0.05$), and immunofluorescence staining further confirmed the elevated MIF expression in advanced (Pfirrmann grade IV) IVDD, relative to those from patients with mild (Pfirrmann grade II) IVDD (Fig. 1A-C). In the rat IVDD model, MRI images showed obvious disc degeneration in IVDD rats compared with control rats (Fig. 1D). H&E staining, Safranin O (SO) staining, and immunohistochemistry staining revealed disrupted disc structure, reduced proteoglycan content, and increased MIF expression in IVDD rat discs (Fig. 1E). Consistently, qRT-PCR (Fig. 1F), WB (Fig. 1G), and immunofluorescence (Fig. 1H) results confirmed that MIF mRNA and protein levels were significantly upregulated in NPCs isolated from IVDD rats compared with control rats. To define the functional downstream mediators of MIF in driving IVDD, we performed an integrated analysis combining proteomic profiling of human degenerative intervertebral disc specimens with protein-protein interaction (PPI) network prediction via the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database. We first delineated the proteomic landscape associated with IVDD progression: volcano plots (Figure S1A) and upset analysis (Figure S1B) identified 48 significantly differentially expressed proteins (DEPs) across advancing Pfirrmann grades of IVDD. Functional enrichment analysis (Figure S1C) revealed that these DEPs were

most prominently enriched in the Complement and coagulation cascades pathway, a key signaling axis implicated in degenerative musculoskeletal disorders. PPI network construction via the STRING database further predicted potential interactions between MIF and three prioritized SERPIN proteins (Figure S1D). Parallel quantitative proteomic profiling demonstrated that MIF, SERPIND1, SERPINE1, and SERPINF2 were synchronously and progressively upregulated with worsening IVDD severity (Figure S1E), and Pearson correlation analysis confirmed a robust, statistically significant positive correlation between MIF expression and each of the three SERPIN family members (Figure S1F). To translate these *in silico* and proteomic findings into biologically meaningful validation, we next characterized the expression patterns of SERPIND1, SERPINE1, and SERPINF2 in both clinical human specimens and rat IVDD model. qRT-PCR and WB analyses revealed that both the mRNA and protein levels of all three SERPIN proteins were significantly elevated in samples from patients with grade IV IVDD, relative to those from patients with grade II IVDD (Fig. 1I and J). Consistent with these clinical observations, we recapitulated this expression pattern in rat IVDD model: qRT-PCR and WB validation confirmed robust upregulation of SERPIND1, SERPINE1, and SERPINF2 at both the transcriptional and translational levels in NPCs harvested from IVDD rats, compared with those from sham-operated control animals (Fig. 1K and L). Immunofluorescence staining further corroborated the increased expression of these three SERPIN proteins in degenerative NPCs *in situ* (Fig. 1M).

3.2 MIF binds to SERPIND1, SERPINE1, and SERPINF2 and regulates their expression in NPCs

Having established the coordinated upregulation of MIF and the three SERPIN proteins in degenerative IVDD, we next interrogated the functional regulatory relationship and direct physical interaction between MIF and these SERPIN family members. We first generated MIF-overexpressing (OE-MIF) and MIF-knockdown (sh-MIF) primary rat NPCs, with matched empty vector negative controls (OE-NC and sh-NC, respectively) (Fig. 2A). WB (Fig. 2B) analysis confirmed efficient overexpression and knockdown of MIF at protein level. Subsequent qRT-PCR (Fig. 2C) and WB (Fig. 2D) analysis revealed that enforced MIF expression significantly upregulated the protein levels of SERPIND1, SERPINE1, and SERPINF2 compared with the OE-NC group, whereas MIF silencing markedly downregulated the expression of all three SERPIN proteins relative to the sh-NC control. These data establish that MIF positively regulates the expression of SERPIND1, SERPINE1, and SERPINF2 in NPCs. To validate the direct physical interaction between MIF and the three SERPIN proteins, we performed orthogonal co-immunoprecipitation (Co-IP) assays. Forward Co-IP using an anti-MIF antibody demonstrated that SERPIND1, SERPINE1, and SERPINF2 were specifically co-precipitated with endogenous MIF, with no non-specific binding detected in the isotype IgG control group (Fig. 2E). Reciprocal Co-IP assays further confirmed this interaction: immunoprecipitation with antibodies targeting SERPIND1, SERPINE1, or SERPINF2 consistently pulled down MIF in NPC lysates (Fig. 2F). Finally, we used surface plasmon resonance (SPR) to quantify the direct binding affinity between recombinant MIF and each SERPIN protein. SPR analysis revealed that MIF bound to SERPIND1, SERPINE1, and SERPINF2 with distinct binding affinities: the equilibrium dissociation constant (K_d) for the MIF-SERPINE1 interaction was the

lowest (12.8 nM), indicating the strongest binding affinity, followed by SERPIND1 (42.5 nM) and SERPINF2 (78.3 nM) (Fig. 2G).

3.3 MIF mediates the PI3K-Akt/NF- κ B pathway via SERPIND1/SERPINE1/SERPINF2 axis in IVDD NPCs

To further dissect the downstream signaling axis mediating the pro-degenerative effect of the MIF-SERPIN regulatory network in IVDD, we performed single-cell RNA sequencing (scRNA-seq) reanalysis of the public GSE165722 dataset. Unsupervised clustering (Figure S2A) and volcano plots (Figure S2B) showed that NPCs derived from intervertebral discs of different Pfirrmann IVDD grades formed distinct cell clusters. Functional enrichment analysis of genes specifically upregulated in degenerative (grade IV) NPCs identified the PI3K-Akt signaling pathway as the most significantly enriched pathway, with 88 differentially expressed genes mapped to this core degenerative axis (Figure S2C). Among these 88 PI3K-Akt pathway-associated genes, we prioritized 7 key hub genes (RELA, NFKB1, MCL1, LAMB3, FLT1, KRAS, and EREG) whose expression was synchronously and progressively upregulated with worsening IVDD severity (Figure S2D).

We next validated that the three SERPIN family members directly regulate the expression of these PI3K-Akt pathway hub genes in NPCs. We performed knockdown and overexpression of SERPIND1, SERPINE1 and SERPINF2 in NPCs isolated from a rat IVDD model (Fig. 3A and B). qRT-PCR (Fig. 3C) and WB (Fig. 3D) analysis revealed that enforced overexpression of SERPIND1, SERPINE1, or SERPINF2 significantly upregulated the protein levels of RELA, NFKB1, MCL1, LAMB3, FLT1, KRAS, and EREG. Conversely, shRNA-mediated knockdown of each individual SERPIN protein markedly downregulated the expression of all 7 hub genes. Building on these findings, we performed rescue experiments to confirm that the MIF-SERPIN axis exerts its regulatory effect via the PI3K-Akt signaling pathway, the core downstream effector axis we identified via scRNA-seq. qRT-PCR (Fig. 3E) and WB (Fig. 3F) analysis demonstrated that OE-MIF significantly upregulated the protein levels of the 7 PI3K-Akt pathway hub genes compared with the OE-NC control group. Critically, co-transfection of OE-MIF with sh-SERPIND1, sh-SERPINE1, or sh-SERPINF2 markedly abrogated the MIF-induced upregulation of these hub genes, compared with the OE-MIF alone group.

3.4 MIF and SERPIN family members regulate pro-inflammatory cytokine production in IVDD NPCs

To further confirm the binding specificity, we performed molecular docking and mutational analysis. Molecular docking results showed clear binding sites between MIF and each of the three SERPIN proteins (Fig. 4A, Table S1). Co-IP results showed that mutation of MIF (deletion of amino acids 105–111) significantly reduced its binding to SERPIND1, SERPINE1, and SERPINF2 (Fig. 4B), and SPR results further verified this finding, with K_d values increased to 61.6 nM (MIF-SERPIND1), 18.6 nM (MIF-SERPINE1), and 113.5 nM (MIF-SERPINF2) after MIF mutation (Fig. 4C). Additionally, WB results confirmed that OE-MIF significantly upregulated the protein expression of SERPIND1, SERPINE1, and SERPINF2, whereas MIF mutation markedly abrogated this promotive effect of MIF on the three SERPIN

family members (Fig. 4D). Collectively, these results demonstrate that MIF directly binds to SERPIND1, SERPINE1, and SERPINF2 and positively regulates their expression, with the strongest binding affinity to SERPINE1. Flow cytometry analysis revealed that MIF overexpression significantly increased the percentage of TNF- α -positive cells compared to the OE-NC control, whereas MIF mutation completely abrogated this pro-inflammatory effect (Fig. 4E). Rescue experiments showed that co-transfection of OE-MIF with sh-SERPIND1, sh-SERPINE1, or sh-SERPINF2 significantly reversed the OE-MIF-induced increase in IL-1 β and TNF- α levels; conversely, co-transfection of sh-MIF with OE-SERPIND1, OE-SERPINE1, or OE-SERPINF2 reversed the sh-MIF-induced decrease in these cytokines (Figure S3A and B). Moreover, inhibition of the NF- κ B pathway with BAY 11-7082 (NF- κ B inhibitor) significantly reversed the increase in IL-1 β and TNF- α levels induced by MIF and SERPIN overexpression (Figure S3C and D). Collectively, these results confirm that MIF-induced pro-inflammatory cytokine production depends on its functional activity and is mediated by SERPIND1/SERPINE1/SERPINF2 via the NF- κ B pathway.

3.5 Screening and biophysical characterization of MIF-specific ligands from *Eucommia ulmoides*

To screen natural MIF-specific ligands from *Eucommia ulmoides*, we first constructed an immobilized MIF column. SDS-PAGE (Fig. 5A) and WB (Fig. 5B) results confirmed successful expression and purification of Tag9-MIF. Streptavidin-modified silica gels were used as the solid-phase matrix. Through the high-affinity specific binding between streptavidin and the Tag9 tag, the Tag9-tagged recombinant MIF (Tag9-MIF) was directionally and stably anchored on the surface of silica microspheres, finally generating MIF-coated gels to establish the affinity screening system for MIF-specific natural ligands (Fig. 5C). X-ray photoelectron spectroscopy (XPS) analysis showed that compared with naked silica gels, MIF-coated silica gels had increased nitrogen and carbon content, confirming successful immobilization of MIF (Fig. 5D-F). Chromatographic analysis showed that known MIF ligands (MIF098, ISO-1, BTZO-1) could specifically bind to the MIF-immobilized column (Fig. 5G), verifying the feasibility of the screening system. Using this system, we screened *Eucommia ulmoides* extract and identified two potential MIF-specific ligands: alphitolic acid and 3-indoleacrylic acid (Fig. 5H and I). We then performed ITC and SPR to characterize their binding to MIF. ITC results showed that both compounds exhibited exothermic binding to MIF, and the thermodynamic parameters showed that alphitolic acid had a lower K_d value (1.85 μ M) than 3-indoleacrylic acid (2.38 μ M), indicating higher binding affinity to MIF. Additionally, alphitolic acid had a more negative ΔG value (-32.5 kJ/mol) than 3-indoleacrylic acid (-30.2 kJ/mol), suggesting more stable binding (Fig. 5J and K). SPR results further confirmed the dose-dependent binding of alphitolic acid and 3-indoleacrylic acid to MIF, with alphitolic acid showing stronger binding affinity (Fig. 5L and M). We first evaluated the effect of alphitolic acid on MIF expression in nucleus pulposus cells (NPCs) isolated from rat intervertebral disc degeneration (IVDD) models. CCK-8 assay was performed to determine the non-cytotoxic concentration range of alphitolic acid, with IC_{50} values of 12.57 μ M (24 h), 10.06 μ M (48 h), and 7.37 μ M (72 h) (Figure S4A). Subsequent experiments were performed using concentrations $\leq$ 16 μ M to avoid non-specific cytotoxicity. qRT-PCR and Western blot analysis demonstrated that alphitolic acid downregulated MIF mRNA and protein expression in both dose- and time-dependent manners (Figure S4B and C). To investigate the functional consequences of

MIF inhibition by aliphatic acid, we examined the expression of extracellular matrix (ECM) metabolism-related genes in NPCs. Overexpression of MIF significantly downregulated the expression of collagen type II (Col-2), a major structural component of the nucleus pulposus, and upregulated matrix metalloproteinase 13 (MMP-13), a key enzyme responsible for ECM degradation. Treatment with aliphatic acid reversed these effects in a dose-dependent manner: Col-2 expression was gradually restored, while MMP-13 expression was correspondingly reduced with increasing aliphatic acid concentrations (Fig. 5N). These results indicate that aliphatic acid can attenuate MIF-mediated ECM degradation in NPCs.

3.6 Characterization of aliphatic acid-loaded liposome hydrogel scaffold (Alp@HDgel) and its therapeutic effects in a rat IVDD model

To enhance the bioavailability and local retention of aliphatic acid, we prepared aliphatic acid-loaded liposomes and constructed an injectable composite hydrogel (Alp@HDgel). TEM images showed that both empty liposomes and aliphatic acid-loaded liposomes had a regular spherical morphology with a particle size of ~ 200 nm (Fig. 6A). SEM images showed that the blank hydrogel and aliphatic acid-liposome-loaded hydrogel had a porous, interconnected microstructure, which is conducive to nutrient exchange and drug release (Fig. 6B). UV-Vis absorption spectra showed a specific absorption peak of aliphatic acid in the aliphatic acid-loaded liposomes, confirming successful loading of aliphatic acid (Fig. 6C). In vitro release experiments showed that aliphatic acid was released from liposomes in a sustained manner, with cumulative release reaching $80.2 \pm 3.1\%$ over 48 h (Fig. 6D). In vitro degradation experiments showed that the hydrogel scaffold degraded gradually over 10 days, with $61.3 \pm 2.7\%$ of the weight remaining on day 10, indicating good biodegradability (Fig. 6E). Stress-strain curves showed that the hydrogel had good tensile and compressive properties, which can withstand the mechanical stress of the intervertebral disc microenvironment (Fig. 6F). These results indicate that the Alp@HDgel has excellent physicochemical properties and sustained drug release capability, making it suitable for local drug delivery in IVDD treatment.

We further evaluated the in vivo therapeutic effect of Alp@HDgel in a puncture-induced rat IVDD model. The animal experiment design is shown in Fig. 6G: the rat IVDD model was established by needle puncture, and intradiscal injection of Alp@HDgel was performed 1 week post-injury, with therapeutic effects evaluated at 8 weeks post-treatment. ELISA analysis revealed that intradiscal administration of Alp@HDgel markedly downregulated the protein expression of two core pro-inflammatory cytokines driving IVDD progression, IL-1 β and TNF- α , in degenerative disc tissues at 8 weeks after treatment. Compared with the untreated IVDD model group, Alp@HDgel treatment induced a robust and significant reduction in both inflammatory factors; critically, this anti-inflammatory efficacy was significantly more potent than that achieved by blank HD gel or free aliphatic acid alone (Fig. 6H). MRI results showed that compared with the IVDD group, the Alp@HDgel group showed significant improvement in disc signal intensity at 8 weeks post-treatment, which was superior to the HD gel group and the free aliphatic acid group (Fig. 6I). H&E staining showed that the IVDD group had disrupted disc structure, while the Alp@HDgel group had significantly improved disc morphology, with more intact nucleus pulposus and

annulus fibrosus structure (Fig. 6J). Safranin O staining showed that the Alp@HDgel group had significantly increased proteoglycan content compared with the IVDD group, indicating restored ECM integrity (Fig. 6J). Immunofluorescence staining showed that the Alp@HDgel group had significantly increased Col-2 (anabolic marker) expression and decreased MMP-13 (catabolic marker) expression compared with the other groups, confirming that Alp@HDgel effectively restores the balance between ECM anabolism and catabolism (Fig. 6K). Collectively, these results demonstrate that Alp@HDgel can significantly reverse disc degeneration in vivo, exerting a superior therapeutic effect compared with free aliphatic acid.

4. Discussion

4.1 Key findings and mechanistic insights

IVDD is a complex degenerative disease driven by multiple factors, and identifying key pathogenic targets is crucial for developing effective therapeutic strategies. In this study, we identified MIF as a critical pro-degenerative target in IVDD, which is significantly overexpressed in degenerated NPCs from both clinical patients and animal models. This is consistent with previous studies showing that MIF is involved in various degenerative diseases, such as osteoarthritis and intervertebral disc herniation[7, 8, 18, 19]. However, the specific molecular mechanisms by which MIF regulates IVDD progression remain unclear. To the best of our knowledge, this is the first report that MIF directly binds to SERPIND1, SERPINE1, and SERPINF2 (SERPIN family members) and positively regulates their expression, with the strongest binding affinity to SERPINE1. This finding sheds new mechanistic light on MIF-mediated IVDD progression.

The PI3K-Akt/NF- κ B pathway is a key signaling pathway involved in inflammation, cell proliferation, and ECM degradation, and its abnormal activation is closely associated with IVDD progression[20–22]. Our results demonstrated that MIF activates the PI3K-Akt/NF- κ B signaling axis through the regulation of SERPIND1, SERPINE1, and SERPINF2, thereby upregulating the pro-inflammatory cytokines IL-1 β and TNF- α . SERPINE1, a member of the SERPIN family, is a serine protease inhibitor that regulates plasminogen activation and ECM remodeling[23]. Previous studies have shown that SERPINE1 is overexpressed in IVDD and promotes disc degeneration by inducing inflammation and ECM degradation[11, 24]. Our results show that MIF has the strongest binding affinity to SERPINE1, and SERPINE1-mediated PI3K-Akt/NF- κ B pathway activation is the key mechanism by which MIF promotes IVDD. This suggests that SERPINE1 may be a potential therapeutic target downstream of MIF, and targeting the MIF-SERPINE1 axis may be an effective strategy for IVDD treatment.

4.2 Screening and evaluation of MIF-specific ligands from *Eucommia ulmoides*

Traditional Chinese herbs have been widely used in IVDD treatment due to their multi-target and low-toxicity advantages. *Eucommia ulmoides*, a classic TCH, has been reported to have anti-inflammatory

and bone-protective effects, but its specific bioactive components and molecular targets remain unclear[25, 26]. In this study, we used immobilized MIF chromatography to screen MIF-specific ligands from *Eucommia ulmoides*, which is a powerful method for identifying target-specific natural products. We identified two potential MIF ligands: alphitolic acid and 3-indoleacrylic acid. Biophysical characterization using ITC and SPR showed that alphitolic acid has higher binding affinity to MIF than 3-indoleacrylic acid, indicating that alphitolic acid is a more promising MIF inhibitor.

Alphitolic acid is a triterpenoid compound isolated from *Eucommia ulmoides*, which has been reported to have anti-inflammatory, antioxidant, and anti-tumor effects[27–29]. Our results show that alphitolic acid can effectively bind to MIF and alleviate MIF-induced inflammation and ECM degradation, which is consistent with its reported anti-inflammatory effects. This finding not only identifies a new bioactive component of *Eucommia ulmoides* but also provides a natural MIF inhibitor for IVDD treatment.

4.3 Advantages of Alp@HDgel in IVDD treatment

The avascular nature of the intervertebral disc makes it difficult for systemic drug delivery to achieve effective local drug concentration, limiting the therapeutic effect of drugs. Injectable hydrogels have emerged as ideal carriers for local drug delivery in IVDD treatment due to their excellent biocompatibility, shear-thinning property, and sustained release capability[13, 30]. In this study, we constructed an injectable composite hydrogel (Alp@HDgel) using HA and DA, which are biocompatible and biodegradable materials. HA is a major component of the intervertebral disc ECM, which can provide a suitable microenvironment for NPC survival and ECM repair[31, 32]. DA has adhesive properties, which can enhance the retention of the hydrogel in the intervertebral disc[33]. Our results show that Alp@HDgel has a porous, interconnected microstructure, good mechanical properties, and sustained drug release capability. In vivo experiments show that Alp@HDgel significantly reverses disc degeneration, restores ECM integrity, and inhibits inflammation in a rat IVDD model, outperforming free alphitolic acid treatment. This is because Alp@HDgel can sustainably release alphitolic acid in the intervertebral disc, maintain effective local drug concentration, and avoid the rapid clearance of free drugs. Additionally, the hydrogel can provide mechanical support for the degenerated disc and promote ECM repair, further enhancing the therapeutic effect.

4.4 Limitations and future perspectives

Despite the significant findings of this study, there are several limitations that need to be addressed in future research. First, this study focused on the regulatory role of MIF and SERPIN family members in NPCs, but the intervertebral disc is a complex tissue composed of multiple cell types (e.g., annulus fibrosus cells, chondrocytes, and immune cells). Future studies should explore the role of the MIF-SERPINE1-PI3K-Akt/NF- κ B axis in other cell types of the intervertebral disc to fully understand its regulatory network in IVDD progression. Second, the animal model used in this study is a puncture-induced acute IVDD model, which does not fully recapitulate the chronic, age-related degenerative process in humans. Future studies should establish a chronic IVDD model (e.g., aging-induced or mechanical stress-induced model) to verify the therapeutic effect of Alp@HDgel in a more clinically

relevant scenario. Third, the in vivo experiments were performed in rats, and the translational potential of Alp@HDgel in humans needs to be further verified in large animal models (e.g., rabbits or non-human primates) before clinical application. Fourth, although alphitolic acid showed good binding affinity to MIF and therapeutic effects in vitro and in vivo, its pharmacokinetic properties and potential side effects need to be systematically evaluated in future preclinical studies.

In future research, we will focus on the following aspects: (1) Explore the interaction between MIF-SERPINE1 axis and other signaling pathways (e.g., MAPK, Wnt/ β -catenin) in IVDD to further clarify the complex molecular mechanism of IVDD progression; (2) Optimize the formulation of Alp@HDgel, such as adjusting the ratio of HA and DA, or modifying the hydrogel with targeting molecules (e.g., MIF antibodies) to enhance its targeting ability; (3) Combine alphitolic acid with other bioactive components (e.g., other natural products or growth factors) to achieve synergistic therapeutic effects; (4) Conduct preclinical pharmacokinetic and toxicological studies to lay the foundation for the clinical translation of Alp@HDgel.

4.5 Conclusion

In summary, this study first identified MIF as a critical pro-degenerative mediator of intervertebral disc degeneration (IVDD) and delineated the underlying molecular mechanism: MIF accelerates IVDD progression principally through activating the SERPINE1-dependent PI3K-Akt/NF- κ B inflammatory cascade. From *Eucommia ulmoides*, we further screened alphitolic acid as a high-affinity specific ligand targeting MIF with prominent anti-IVDD activity. To achieve local long-term administration, we fabricated an Alp@HDgel hydrogel with favorable physicochemical characteristics and sustained drug release performance; in vivo experiments in rat IVDD models validated that this hydrogel effectively alleviated intervertebral disc degeneration. Collectively, our work establishes a innovative therapeutic strategy for IVDD by targeting the MIF-SERPINE1-PI3K-Akt/NF- κ B signaling axis via natural product-loaded sustained-release hydrogel biomaterials, and highlights the translational value of herbal-derived compounds for degenerative musculoskeletal disorders. Beyond therapeutic development, these findings expand our mechanistic insights into IVDD pathogenesis and offer a promising candidate formulation with great potential for future clinical translation.

Declarations

Ethics approval and consent to participate

All clinical nucleus pulposus specimen collection and related experimental analyses were approved by the Ethics Committee of Tangdu Hospital, The Fourth Military Medical University (Approval No. 202102-16). All enrolled patients provided written informed consent prior to tissue harvesting, and all procedures strictly complied with the principles outlined in the Declaration of Helsinki.

All animal experiments were performed in accordance with the Animal Care and Use Committee of The Fourth Military Medical University (Approval No. 202203-08), the NIH Guide for the Care and Use of

Laboratory Animals, and the ARRIVE 2.0 guidelines. All surgical operations, animal housing, postoperative care and tissue collection were conducted to minimize animal suffering.

Competing interests

The authors declare that they have no known competing financial interests or non-financial conflicts of interest that could influence the work reported in this manuscript. No commercial entities participated in study design, sample collection, data analysis, manuscript writing, or the decision to submit this work for publication.

Author Contribution

Y.H. and Y.K. designed the research, performed clinical sample collection, cellular experiments, molecular binding assays, and drafted the original manuscript. Q.L. completed natural product extraction, HPLC-MS/MS identification and ligand screening. L.L., Y.Q., X.W., J.Y., Z.Z., X.Z., Q.W., S.L. carried out hydrogel fabrication, material characterization, in vitro release and degradation tests, and animal model construction. B.L. conceived the whole project, supervised all experiments, revised the manuscript, and provided financial support. All authors read and approved the final submitted manuscript.

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Data Availability

Data are available from the corresponding author upon reasonable request. The relevant raw data will be deposited in public data repositories for open access if required by the editorial office.

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Figures

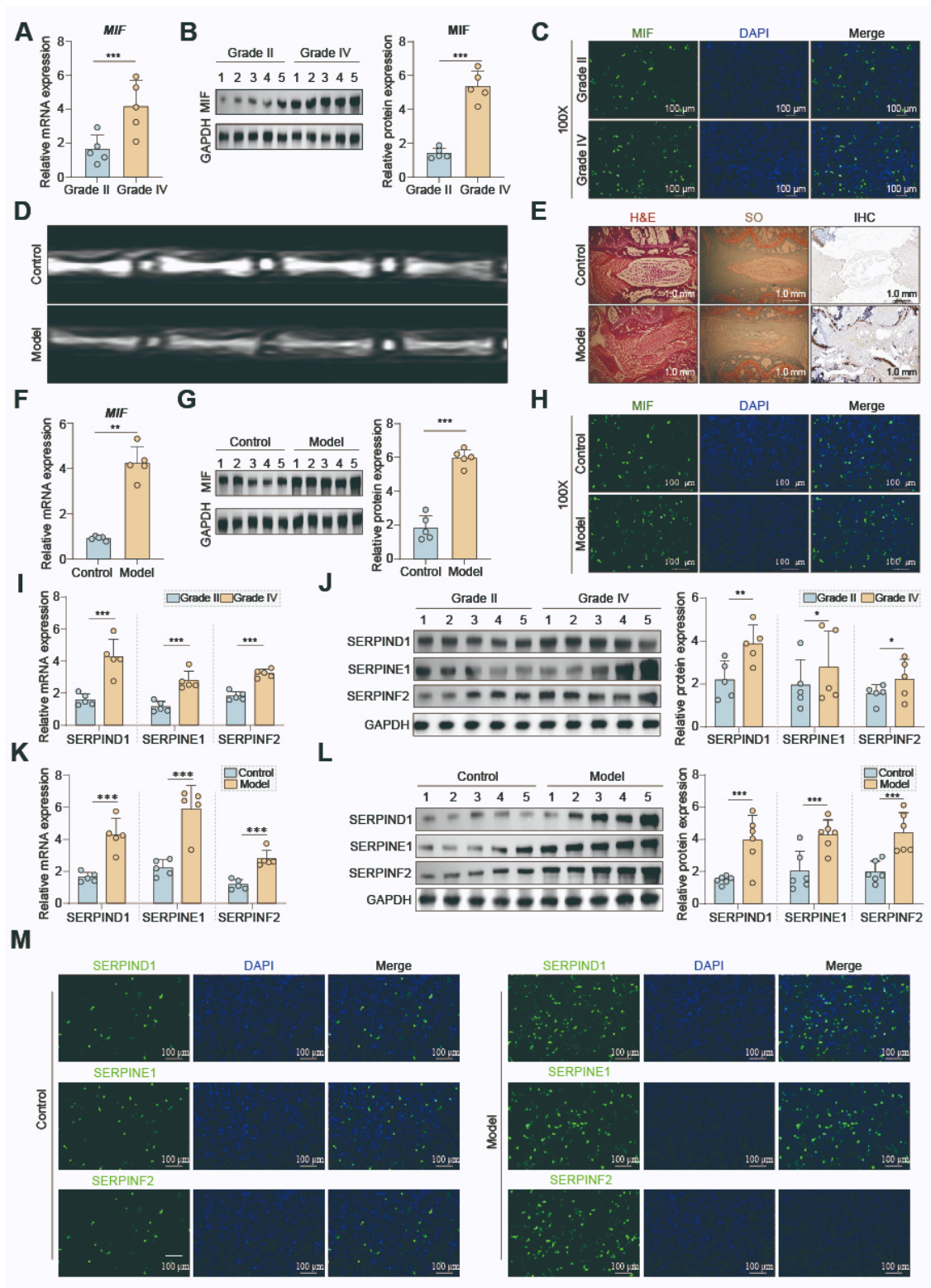


Figure 1

MIF is significantly overexpressed in IVDD NPCs

(A) qRT-PCR results showing the relative mRNA expression level of MIF in nucleus pulposus cells (NPCs) isolated from patients with Grade II and Grade IV intervertebral disc degeneration (IVDD) (n=5 per group). (B) Western blotting (WB) results showing the protein expression level of MIF in NPCs from Grade II and

Grade IV IVDD patients (n=5 per group). (C) Immunofluorescence staining images of MIF in NPCs from Grade II and Grade IV IVDD patients. (D) T2-weighted MRI images of intervertebral discs in control rats and IVDD model rats, showing the degree of disc degeneration. (E) Hematoxylin-eosin (H&E) staining, Safranin O (SO) staining, and immunohistochemistry (IHC) staining of MIF in intervertebral disc tissues from control and IVDD rats (n=5 per group); H&E staining shows tissue morphology, SO staining reflects proteoglycan content, and IHC staining shows MIF protein localization. (F) qRT-PCR results showing the relative mRNA expression of MIF in NPCs from control and IVDD rats (n=5 per group). (G) WB results showing the protein expression of MIF in NPCs from control and IVDD rats (n=5 per group). (H) Immunofluorescence staining images of MIF in NPCs from control and IVDD rats. (I) qRT-PCR results showing the relative mRNA expression levels of SERPIND1, SERPINE1, and SERPINF2 in NPCs from Grade II and Grade IV IVDD patients (n=5 per group). (J) WB results showing the protein expression levels of SERPIND1, SERPINE1, and SERPINF2 in NPCs from Grade II and Grade IV IVDD patients (n=5 per group). (K) qRT-PCR results showing the relative mRNA expression levels of SERPIND1, SERPINE1, and SERPINF2 in NPCs from control and IVDD rats (n=5 per group). (L) WB results showing the protein expression levels of SERPIND1, SERPINE1, and SERPINF2 in NPCs from control and IVDD rats (n=5 per group). (M) Immunofluorescence staining images of SERPIND1, SERPINE1, and SERPINF2 in NPCs from control and IVDD rats. Notes: Data are presented as mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001.

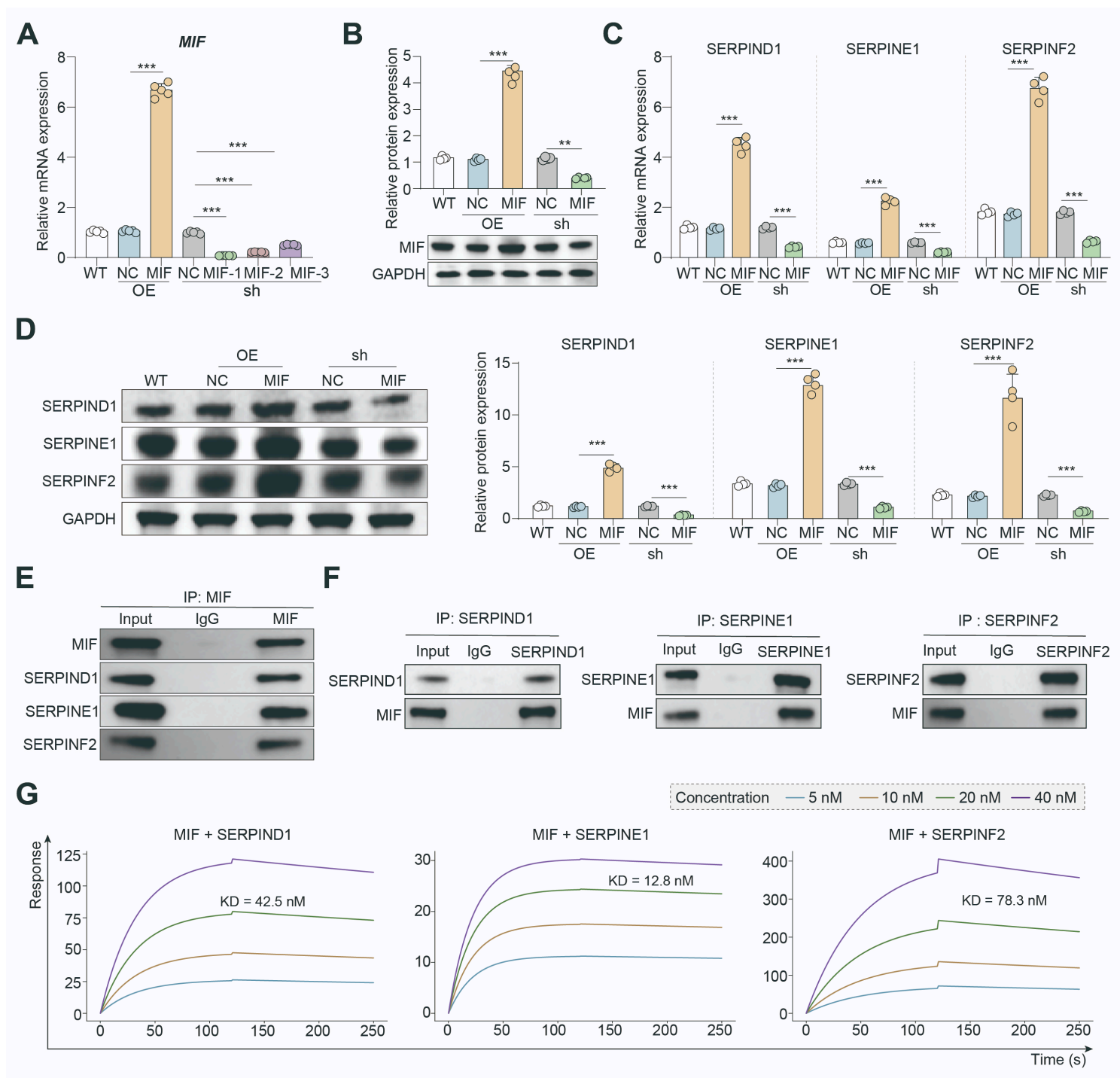


Figure 2

MIF binds to SERPIND1, SERPINE1, and SERPINF2 and regulates their expression

(A) qRT-PCR analysis of MIF mRNA expression in wild-type (WT), empty vector control (OE-NC), MIF-overexpressing (OE-MIF), and MIF-knockdown (sh-MIF-1/2/3) primary rat NPCs; sh-MIF-1/2/3 are three different siRNA sequences targeting MIF. (B) WB results showing the protein expression level of MIF in primary rat NPCs with MIF silencing (sh-MIF) or overexpression (OE-MIF); empty vector (sh-NC/OE-NC) was used as the negative control. (C) Relative mRNA expression levels of SERPIND1, SERPINE1, and SERPINF2 in the indicated cell groups (WT, OE-NC, OE-MIF, sh-NC, sh-MIF); sh-NC is the negative control

for sh-MIF. (D) WB results showing the protein expression levels of SERPIND1, SERPINE1, and SERPINF2 in MIF-silenced or MIF-overexpressed primary rat NPCs. (E) Co-immunoprecipitation (co-IP) assay showing the interaction between MIF and SERPIND1, SERPINE1, or SERPINF2; cell lysates were immunoprecipitated with anti-MIF antibody or IgG (negative control), and then immunoblotted with antibodies against MIF, SERPIND1, SERPINE1, and SERPINF2. (F) Reciprocal co-IP assays confirming the specific interaction between MIF and each SERPIN family protein; cell lysates were immunoprecipitated with anti-SERPIND1, anti-SERPINE1, or anti-SERPINF2 antibodies, respectively, followed by immunoblotting with antibodies against MIF and the corresponding SERPIN protein. (G) Surface plasmon resonance (SPR) results showing the binding affinity between MIF and SERPIND1, SERPINE1, or SERPINF2; the dissociation constants (K_d) were calculated as follows: MIF-SERPIND1 (42.5 nM), MIF-SERPINE1 (12.8 nM, the lowest K_d), and MIF-SERPINF2 (78.3 nM). Notes: Data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

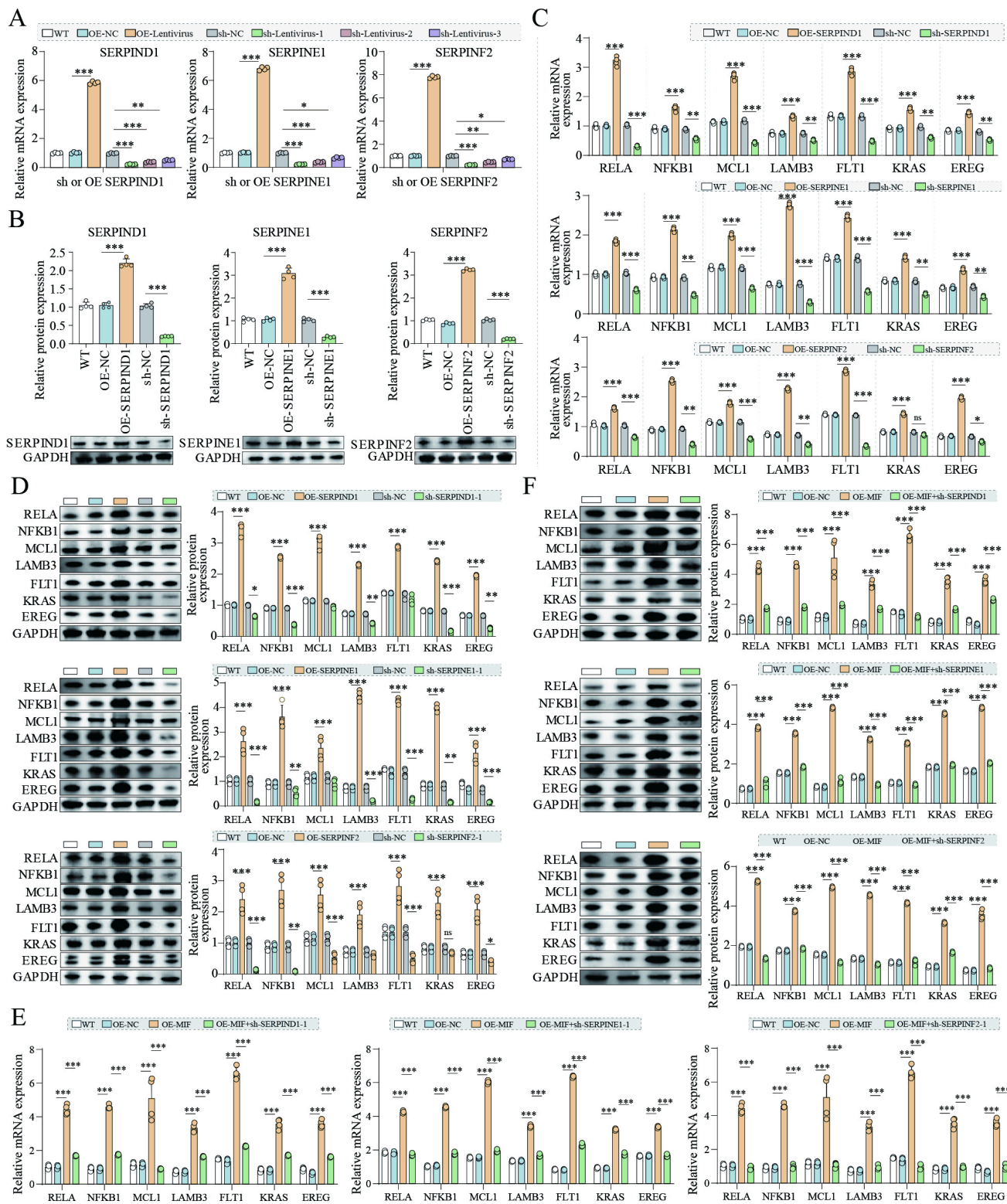


Figure 3

MIF mediates the PI3K-Akt/NF- κ B pathway via SERPIND1/SERPINE1/SERPINF2 axis in IVDD NPCs

(A) Validation of SERPIND1, SERPINE1, or SERPINF2 overexpression and knockdown efficiency by qRT-PCR. (B) WB was performed to validate the efficiency of overexpression and knockdown of SERPIND1, SERPINE1, and SERPINF2 in NPCs. (C) qRT-PCR and (D) WB results demonstrated that knockdown of

SERPIND1, SERPINE1, or SERPINF2 significantly inhibited the expression of PI3K-Akt/NF-κB pathway-related genes (RELA, NFKB1, MCL1, LAMB3, FLT1, KRAS, and EREG), whereas overexpression of these SERPIN proteins markedly promoted their expression. (E) qRT-PCR and (F) WB results showed that MIF overexpression significantly upregulated the expression of the PI3K-Akt/NF-κB pathway-related genes. Notably, compared with the OE-MIF group, co-transfection of OE-MIF with shRNA targeting SERPIND1, SERPINE1, or SERPINF2 significantly abrogated this promotive effect and suppressed the expression of the pathway-related genes. Notes: Data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

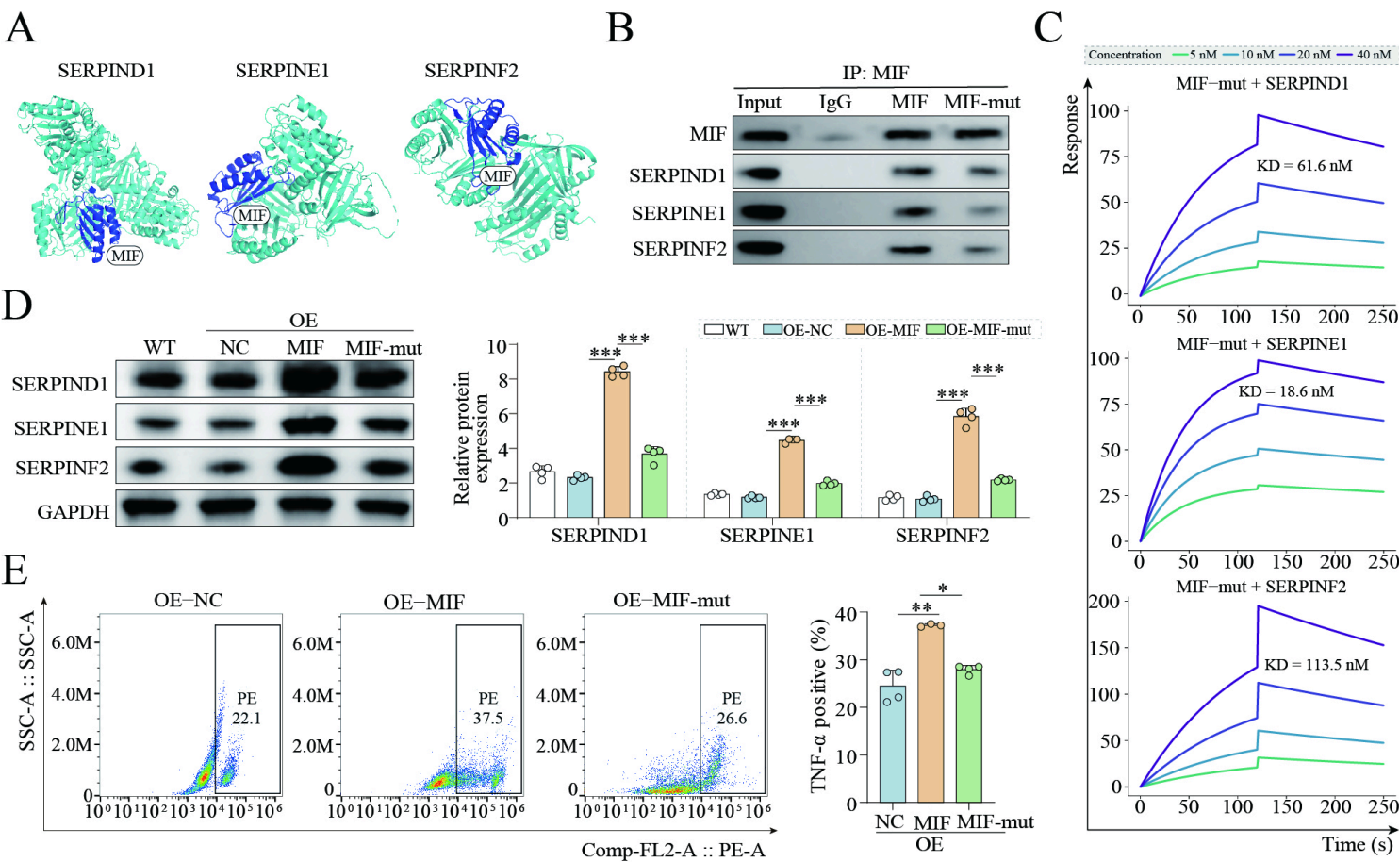


Figure 4

Mutation of MIF reduces its binding to SERPIND1, SERPINE1, and SERPINF2

(A) Molecular docking results showing the binding sites and interaction modes between MIF and SERPIND1, SERPINE1, or SERPINF2. (B) Co-IP results confirming the binding between MIF and SERPIND1, SERPINE1, and SERPINF2; the binding affinity was significantly reduced after MIF mutation (OE-MIF-mut). (C) SPR results further confirming the co-IP findings; the Kd values of MIF mutation binding to SERPIND1, SERPINE1, and SERPINF2 were increased (indicating decreased binding affinity). (D) WB results showed that, compared with the OE-NC group, the protein levels of SERPIND1, SERPINE1, and SERPINF2 were significantly upregulated in the OE-MIF group. In contrast, this promotive effect of MIF on these genes was markedly attenuated by MIF mutation. (E) Flow cytometry analysis revealed that MIF overexpression significantly increased the percentage of TNF-α-positive cells compared to the OE-

NC control, whereas MIF mutation completely abrogated this pro-inflammatory effect. Notes: All data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

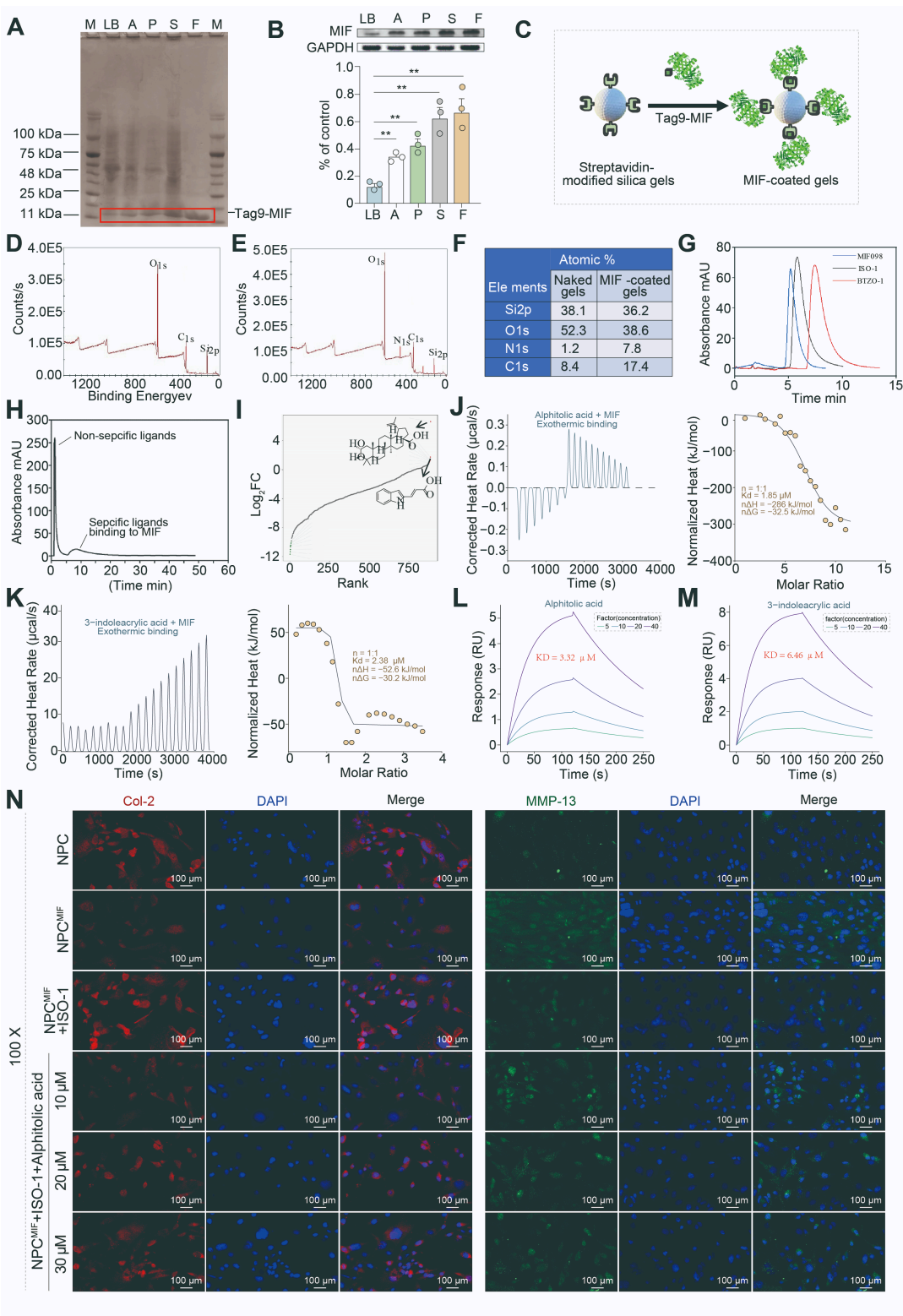


Figure 5

Biophysical characterization of the interaction between aliphatic acid or 3-indoleacrylic acid and MIF

SDS-PAGE analysis of the expression and purification of Tag9-MIF; Lane M: molecular weight marker; LB: bacterial lysate without induction; A: supernatant after induction; P: precipitate after induction; S: flow-through during purification; F: purified Tag9-MIF; the red box indicates the target protein band corresponding to the theoretical molecular weight of Tag9-MIF. (B) Western blot analysis of MIF expression (top panel) and quantitative analysis (bottom panel, $n=3$); GAPDH was used as the loading control. (C) Schematic illustration of MIF immobilization on streptavidin-modified silica gels via the high-affinity interaction between Tag9 and streptavidin. (D, E) X-ray photoelectron spectroscopy (XPS) survey spectra of naked silica gels (D) and MIF-coated silica gels (E), showing the element composition on the gel surface. (F) Atomic percentages of elements on the surface of naked and MIF-coated silica gels, showing increased nitrogen (N) and carbon (C) content after MIF immobilization (consistent with the elemental composition of MIF protein). (G) Chromatographic retention profiles of known MIF ligands (MIF098, ISO-1, BTZO-1) on the MIF-immobilized column, demonstrating specific binding (retention time longer than void time). (H) Chromatographic separation of *Eucommia ulmoides* extract using the MIF-immobilized column, distinguishing non-specific binding components (eluted at void time) and specific binding components (retained and eluted later). (I) Volcano plot of HPLC-MS/MS differential analysis of retained ligands, identifying alphitolic acid (Alp) and 3-indoleacrylic acid as potential MIF-specific binders. (J, K) Isothermal titration calorimetry (ITC) analysis: Representative ITC thermograms (upper panels) and binding isotherms (lower panels) for the interaction between MIF and alphitolic acid (J) or 3-indoleacrylic acid (K). Both compounds exhibited exothermic binding to MIF. The key thermodynamic parameters, including the binding stoichiometry (n), dissociation constant (K_d), binding enthalpy (ΔH), and Gibbs free energy (ΔG), are indicated in the figures. (L, M) Surface plasmon resonance (SPR) analysis: Sensorgrams showing the dose-dependent binding of alphitolic acid (L) or 3-indoleacrylic acid (M) to immobilized MIF at different concentrations (5, 10, 20, and 40 μM). The equilibrium dissociation constants (K_d) were calculated based on the binding response values. RU, response units. (N) Col-2 expression was significantly decreased, while MMP-13 expression was notably increased in NPCMIF (OE-MIF) versus normal NPC. Alphitolic acid treatment at graded concentrations restored Col-2 expression and suppressed MMP-13 expression in a dose-dependent fashion in NPCMIF.

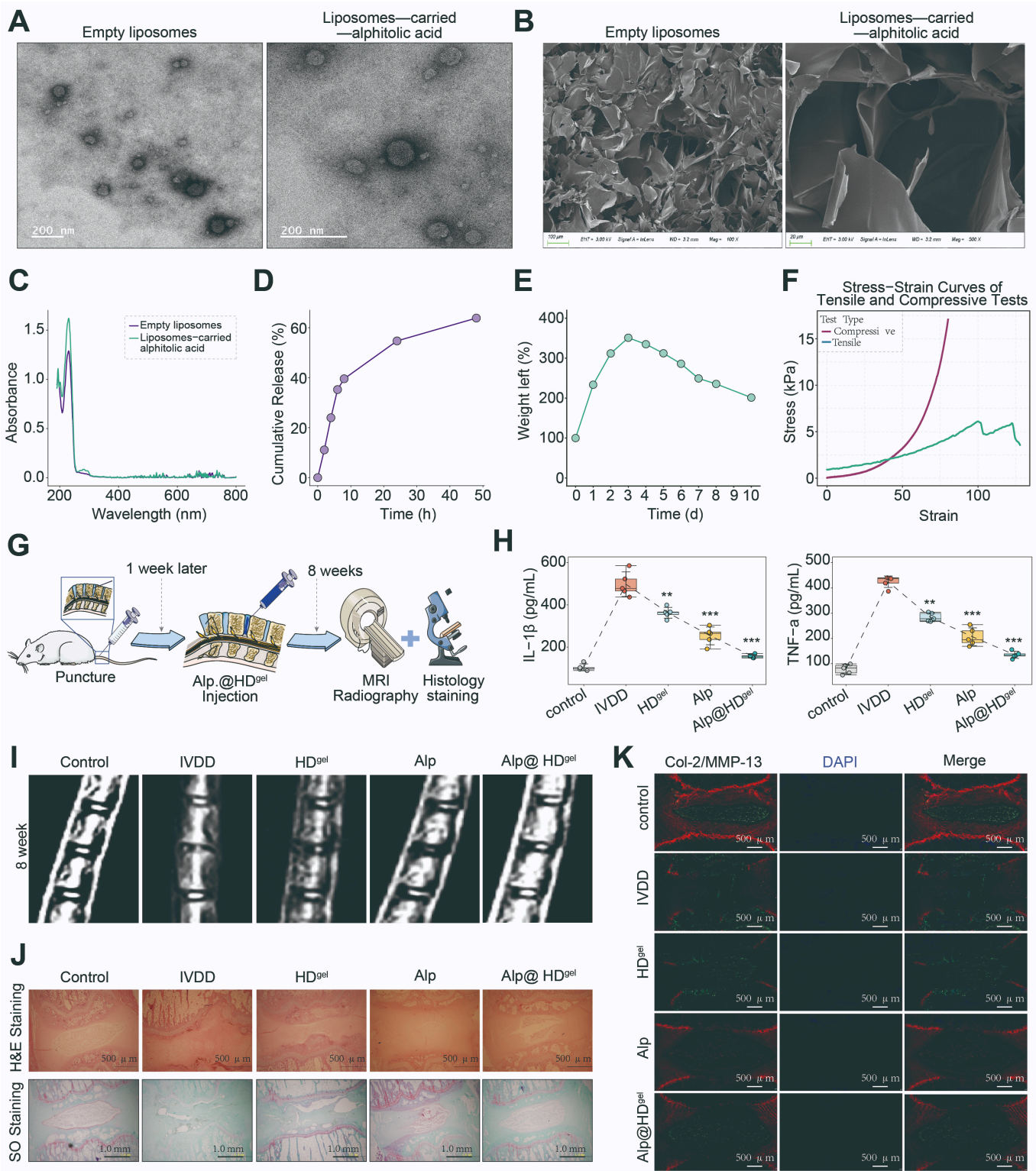


Figure 6

Engineering, physicochemical and functional characterization of Alp@HDgel and its therapeutic effects against IVDD in a rat model

(A) Transmission electron microscopy (TEM) images of empty liposomes (left) and aliphatic acid-loaded liposomes (right), showing the typical spherical morphology of the vesicles; scale bars = 200 nm.

(B) Scanning electron microscopy (SEM) images of the blank hydrogel (left, 100× magnification, scale bar = 100 μm) and aliphatic acid-liposome-loaded hydrogel (right, 500× magnification, scale bar = 20 μm), illustrating the porous and interconnected microstructure of the hydrogel scaffolds, which is conducive to nutrient exchange and drug release. (C) UV-Vis absorption spectra of empty liposomes (blue line) and aliphatic acid-loaded liposomes (red line); the characteristic absorption peak of aliphatic acid in the red line confirms the successful loading of aliphatic acid into liposomes. (D) In vitro cumulative release profile of aliphatic acid from liposomes over 48 hours ($n = 3$), showing the sustained release characteristic of aliphatic acid. (E) In vitro degradation behavior of the hydrogel scaffold, presented as the percentage of remaining weight over 10 days ($n = 3$), reflecting the degradation rate of the scaffold in vitro. (F) Representative stress-strain curves of the hydrogel under tensile (cyan line) and compressive (purple line) loading conditions, indicating the mechanical properties of the hydrogel scaffold. (G) Schematic illustration of the animal experiment design: The rat IVDD model was established by needle puncture; 1 week after injury, intradiscal injection of Alp@HD hydrogel (aliphatic acid-liposome-loaded hydrogel) was performed. The therapeutic effects were evaluated at 8 weeks after treatment via MRI radiography and histological staining. (H) ELISA quantification of IL-1 β and TNF- α protein levels in disc tissues at 8 weeks post-treatment. (I) Representative T2-weighted MRI images of intervertebral discs in different groups (IVDD model group, Control group, HD gel group, Alp group, Alp@HD gel group) at 8 weeks post-treatment; the signal intensity of the intervertebral disc reflects the degree of disc degeneration. (J) Hematoxylin-eosin (H&E) staining of intervertebral disc tissues showing morphological and structural changes across groups at 8 weeks; normal disc structure was partially restored in the Alp@HD gel group. Safranin O (SO) staining demonstrating proteoglycan content and extracellular matrix integrity in disc tissues at 8 weeks; the SO staining intensity was significantly higher in the Alp@HD gel group, indicating increased proteoglycan content. (K) Immunofluorescence staining of Col-2 (red, an anabolic marker of extracellular matrix) and MMP-13 (green, a catabolic marker of extracellular matrix) in disc tissues, with DAPI (blue) for nuclear counterstaining; the expression of Col-2 was increased and MMP-13 was decreased in the Alp@HD gel group at 8 weeks, indicating the improvement of extracellular matrix metabolism. Notes: All data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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