

miR-27b-3p modulates CD4⁺CD39⁺ Tregs to drive immune-mediated intervertebral disc degeneration

QIUWEI LI^{1-3*}, CHENHAO ZHAO^{1-3*}, PEILIN JIN³ and CAILIANG SHEN^{1,2}

¹Department of Orthopedics and Spine Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230022, P.R. China; ²Laboratory of Spinal and Spinal Cord Injury Regeneration and Repair, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230022, P.R. China; ³Institute of Health and Medicine, Hefei Comprehensive National Science Center, Economic and Technological Development Zone, Hefei, Anhui 230022, P.R. China

Received July 17, 2025; Accepted August 27, 2025

DOI: 10.3892/ijmm.2025.5634

Abstract. Intervertebral disc degeneration (IVDD) is a major cause of chronic back pain, yet its molecular mechanisms remain poorly understood despite its high prevalence. In the present study, the significant role of microRNA (miR)-27b-3p in regulating 731 immune cell types was systematically uncovered utilizing Mendelian randomization (MR) and single-cell RNA sequencing, with a particular focus on CD4⁺CD39⁺ regulatory T cells (Tregs); and its critical impact on immune-mediated IVDD progression was highlighted. A total of 76 miRs were screened and, through MR analysis, a significant causal relationship between miR-27b-3p and IVDD was identified. Subsequent *in vivo* and *in vitro* experiments demonstrated that miR-27b-3p overexpression not only promoted apoptosis of nucleus pulposus cells but also accelerated IVDD by modulating the immune functions of CD4⁺CD39⁺ Tregs. Single-cell RNA sequencing further revealed a marked upregulation of immune-related genes in degenerated discs, particularly those involved in immune cell migration, inflammation and apoptotic regulation pathways. These findings suggest that miR-27b-3p plays a pivotal role in IVDD by influencing various immune cells, especially CD4⁺CD39⁺ Tregs, underscoring its potential as a therapeutic target with significant clinical implications. Further research into the mechanisms of miR-27b-3p could open new avenues for IVDD treatment strategies, offering promising possibilities for future clinical applications.

Introduction

Aging is a fundamental biological process that underlies the progression of various degenerative diseases, including intervertebral disc degeneration (IVDD) (1). IVDD is a leading cause of chronic lower back pain, particularly in older adults, and is characterized by the breakdown of the intervertebral discs (IVD), leading to significant musculoskeletal decline (2). Despite its prevalence, the molecular mechanisms driving IVDD, especially those related to immune system dysregulation, remain poorly understood (3,4). Prevention and treatment of IVDD are challenging because of the high incidence and recurrence rates, and associated expenses (5,6). Current treatment options for IVDD include conservative management with medications and surgical intervention to alleviate compression, although both fail to prevent further degeneration (7,8). Hence, additional research on the pathogenesis of IVDD is imperative to elucidate the underlying mechanisms and develop specific treatment approaches.

It has been recently shown that microRNAs (miRNAs or miRs) play important roles in cell proliferation, apoptosis, pyroptosis, and regulation of cytokine release. In addition, miRNAs have been linked to the onset and progression of IVDD (9). Therefore, the specific mechanisms employed by miRNAs associated with the onset and progression of IVDD must be clarified to develop miRNA-based therapies to prevent disc degeneration. Various miRNAs play therapeutic roles in numerous diseases by controlling inflammatory responses, thus an in-depth understanding of the complex relationship between miRNAs and immunity is crucial for the prevention and treatment of IVDD (10-12). Immune cells, including lymphocytes and various phagocytes, recognize antigens and produce specific immune responses via complex interactions with musculoskeletal cells. T lymphocytes and natural killer cells promote osteoclast formation and a subsequent anabolic response during inflammation resolution (13,14). The IVD has been identified as an immune privileged organ because of the unique avascular structure (15). Impairment causes changes to the internal environment of the IVD, resulting in pro-inflammatory and catabolic responses. Changes to the IVD microenvironment can cause recruitment of immune cells and the release of inflammatory factors, which can induce

Correspondence to: Professor Cailiang Shen, Department of Orthopedics and Spine Surgery, The First Affiliated Hospital of Anhui Medical University, 218 Jixi Road, Hefei, Anhui 230022, P.R. China
E-mail: scl1616@163.com

*Contributed equally

Key words: microRNA-27b-3p, immune modulation, intervertebral disc degeneration, regulatory T cells, single-cell RNA sequencing

apoptosis, senescence, pyroptosis, and degradation of the extracellular matrix (ECM) in the late stage of IVDD (16,17). In addition, infiltration of the IVD by immune cells can induce changes to the miRNA content and subsequent immune responses in the nucleus pulposus (NP) (18-20).

IVDD is increasingly recognized as a key contributor to age-related musculoskeletal decline. As the population ages, understanding the molecular mechanisms underlying IVDD is critical for developing interventions that can mitigate the debilitating effects of this condition. Recent studies have highlighted the role of miRNAs in modulating cellular aging processes, including apoptosis, senescence and immune responses (10-12). However, the specific involvement of miR-27b-3p in these aging-related pathways remains underexplored. The present study aims to fill this gap by investigating the role of miR-27b-3p in IVDD, particularly in the context of its interaction with CD4⁺CD39⁺ Treg cells, which are known to play a role in immune aging.

Mendelian randomization (MR) leverages genetic information to investigate causal relationship between exposure variables and outcome factors (21). Genome-wide analysis is increasingly used in association studies (22). In the present study, the MR approach was used to examine the causal relationship between 76 miRNAs and the risk of IVDD. Then, 731 immune cell features were selected as intermediaries to investigate potential relationships with the complex immune response mechanism of IVDD. In addition, *in vivo* and *in vitro* analyses were conducted to validate the findings. The present study represents a pioneering effort in utilizing MR to elucidate the causal relationship between miR-27b-3p and IVDD. Furthermore, by focusing on the modulation of CD4⁺CD39⁺ Treg cells, the present study offers a unique perspective on the immune mechanisms driving IVDD, positioning miR-27b-3p as a promising therapeutic target.

Materials and methods

Ethics statement. The animal experimental protocols were authorized by the Laboratory Animal Care and Use Committee of the Institute of Health and Medicine, Hefei Comprehensive National Science Center (approval no. IHM-AP-2024-015; Hefei, China).

Study design. The causal relationship between 76 miRNAs in whole blood and low back pain was assessed using a two-sample MR approach, with single nucleotide polymorphisms (SNPs) as instrumental variables (IVs). MR analysis relies on three key assumptions: i) IV independence from confounders, ii) a significant relationship between IV and the exposure, and iii) IV affecting outcomes solely through the exposure (23). To enhance result reliability, heterogeneity sensitivity and reverse MR analyses were performed. In total, 731 immune cells were also selected as mediators to improve understanding of miRNA functions in IVDD. A two-step MR analysis was used to identify the mediating pathway (24). Furthermore, *in vitro* and *in vivo* experiments validated the role of miR-27b-3p in IVDD. Using the GSE205535 dataset from Gene Expression Omnibus database (25), single-cell suspensions were analyzed to identify immune subpopulations, focusing on CD39⁺CD4⁺ regulatory T cells (Tregs). UMAP dimensional reduction and

clustering methods revealed cellular heterogeneity, while differential gene expression analysis highlighted key signaling pathways in immune regulation and disc degeneration. This approach provided new insights into immune cell involvement in IVDD pathogenesis at a single-cell level (Fig. S1). The present study aimed to explore the role of miR-27b-3p in IVDD development and identify potential treatment targets and pathways.

Data sources. Experimental datasets from individuals of European descent were utilized to guarantee the precision of the experiments. miRNA data was collected from a study by Huan *et al* (26) that found miRNA expression quantitative trait loci on a genome-wide scale. The study involved analyzing miR-eQTLs in whole blood samples from 5,239 individuals, leading to the identification of 5,269 cis-miR-eQTLs in 76 mature adults (26). The 731 immune cell types utilized for Mendelian analysis were obtained from the dataset of Orrù *et al* (27), which studied over 3,000 individuals. These data were compiled to facilitate MR analysis. Detailed information on the 731 immune cell types is provided in Table SI. The IDD dataset was obtained from a Finnish database of 218,792 individuals, including 19,509 cases and 199,283 controls. The human data used in the MR analysis were sourced from a well-characterized cohort, ensuring the representativeness of the sample for clinical translation. This cohort provides a robust foundation for exploring the potential of miR-27b-3p as a therapeutic target for IVDD.

Instruments selection. SNPs selected for MR analysis must have a substantial genome-wide correlation and strong association with the exposure of interest. Therefore, a probability (p) value $<1 \times 10^{-5}$ and linkage disequilibrium ($r^2=0.001$, kb=10,000) were used to identify appropriate SNPs while preserving data independence. The PhenoScanner database (<https://github.com/phenoscanner/>) was manually screened to identify and exclude SNPs that might be influenced by confounding factors associated with disc degeneration. The F-value of each SNP was calculated to verify a robust relationship between IVs and exposure consistent with the premise of MR analysis (28,29). The F value was calculated as $[(N-K-1)/k] \times [R^2/(1-R^2)]$, where N is the sample size in a genome-wide association study, K denotes the quantity of SNPs, and R² indicates the percentage of variations present in the unauthorized database and was calculated as $2 \times (1-MAF) \times MAF \times (\beta/SD)$, where MAF is the minor allele frequency, β is the effect size of the allele, and SD (standard deviation) is calculated by multiplying the standard error by the square root of the sample size (N). SNPs with a minor allele frequency $<10\%$ were considered inefficient and removed to ensure that all SNPs substantially contributed to the variance of the associated metabolite.

The false discovery rate (FDR), which was employed to improve the stability and reliability of the MR data, was calculated as (P-value $\times$ Rankmax)/prank). As previously described (30), an FDR of 0.20 was considered to have outstanding dependability.

Inverse variance weighting (IVW) was utilized as the primary analytic approach. $P < 0.05$ indicated a statistically significant causal link between exposures and outcomes. The weighted median, MR-Egger, simple mode, and weighted mode

were employed to enhance the reliability of the findings (31). Directional pleiotropy was assessed using intercept values from MR-Egger regression. The Cochran's Q test was utilized to assess the considerable heterogeneity among the selected IVs. $P < 0.05$ indicated significant heterogeneity, thus a random effects model was utilized to evaluate the causal relationship. Otherwise, a fixed effects model was employed (32). The influence of additional SNPs on IVW estimations was assessed by 'leave-one-out' sensitivity analysis. The data are presented as the OR and 95% confidence interval (CI). $P < 0.05$ was considered statistically significant. The MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO) method was used to re-evaluate the existence of several SNPs. Data analyses were conducted using R software (version 4.3.2; <https://www.r-project.org/>) with the 'Two-Sample-MR' and 'MR-PRESSO' packages.

Cell culture and treatment. NP cells were obtained from Procell Life Science & Technology Co., Ltd. and cultivated in Dulbecco's modified Eagle's medium supplemented with nutrient mixture F-12, 15% fetal bovine serum, and 1% penicillin-streptomycin. The medium was replaced every 3 days.

Western blot analysis. Cells were divided into six groups: Normal control, lipopolysaccharide (LPS), miR-27b-3p, miR-27b-3p inhibition, LPS + miR-27b-3p, and LPS + miR-27b-3p. After culturing, the cells were rinsed three times with phosphate-buffered saline (PBS), detached using a cell scraper, centrifuged at $224 \times g$ at 5°C for 5 min, and subsequently transferred into new Eppendorf tubes. The cells were lysed using radio immunoprecipitation assay buffer and phenyl-methyl-sulfonyl fluoride (Beyotime Institute of Biotechnology). The protein content was quantified using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). The proteins ($10 \mu\text{g}$ per lane) were combined with loading buffer (4:1 v/v), heated for 10 min, separated by electrophoresis (10%), and then electroblotted on to a polyvinylidene difluoride membrane, which was blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature, and then incubated overnight at 4°C with primary antibodies (1:1,000) against aggrecan (cat. no. ab313636; Abcam), Bax (cat. no. ET1603-34; HUABIO), Bcl-2 (cat. no. ET1702-53; HUABIO), COL2A (cat. no. HA722809; HUABIO) and actin (cat. no. AF7018; Affinity Biosciences), followed by incubation with a horseradish-conjugated anti-rabbit secondary antibody (1:10,000; cat. no. S0001; Affinity Biosciences) against immunoglobulin G at room temperature for 90 min. The protein bands were visualized using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology) and imaged with a multifunctional gel imaging system (Bio-Rad Laboratories, Inc.).

Immunofluorescence (IF). Apoptosis was quantified using TUNEL following the instructions provided by the reagent provider. The cells were initially submerged in xylene for two 5-min intervals. The cells were sequentially exposed to gradient ethanol concentrations (100, 95, 90, 80 and 70%) for 3 min each and then washed twice with PBS. The tissues were treated with Proteinase K working solution

for 15-30 min at a temperature range of $21\text{--}37^{\circ}\text{C}$ or with a cell-permeable solution for 8 min, followed by rinsing twice with PBS. The TUNEL reaction mixture was produced, and the treatment group was combined with $50 \mu\text{l}$ of TdT and $450 \mu\text{l}$ of fluorescein-labeled dUTP solution. The negative control (NC) group received $50 \mu\text{l}$ of fluorescein-labeled dUTP solution, whereas the positive control group was initially treated with $100 \mu\text{l}$ of DNase 1. The reaction took place at room temperature, $\sim 37^{\circ}\text{C}$, for 10-30 min, and the subsequent stages mirrored those of the treatment group. After drying the slides, $50 \mu\text{l}$ of TUNEL reaction mixture (or simply $50 \mu\text{l}$ of fluorescein-labeled dUTP solution for the NC group) was applied to the specimen. The reaction took place in a dark, humid chamber at 37°C for 60 min, and then the slides were ready for further use. The cells were washed three times with PBS. A single droplet of PBS was introduced to quantify apoptotic cells using a fluorescence microscope with an excitation wavelength of 450-500 nm and a detection wavelength of 515-565 nm. After drying the slide, $50 \mu\text{l}$ of converter-POD was applied to the specimen. The coverslips or sealing membranes were then attached and the reaction took place in a dark moist box at 37°C for 30 min. Finally, the specimen was washed with PBS three times. Between 50 and 100 microliters of DAB substrate were applied to the tissue, and the reaction was carried out at a temperature between $15\text{--}25^{\circ}\text{C}$ for 10 min. The cells were washed three times with PBS. The slides were counterstained with hematoxylin or methyl green and promptly washed with tap water after a brief period. Dehydrated with a gradient of alcohol, made transparent using xylene, then sealed with neutral gum. A drop of PBS or glycerol was placed in the field of vision 200 to 500 cells were counted using a light microscope, and images were captured.

Detection of $\text{CD4}^{+}\text{CD39}^{+}$ Treg cells. The purpose of this experiment was to detect and quantify $\text{CD4}^{+}\text{CD39}^{+}$ Treg cells in rat models, focusing on their potential role in IVDD. Specifically, it was aimed to identify the presence of $\text{CD4}^{+}\text{CD39}^{+}$ Treg cells in the NP tissue and their interaction with miR-27b-3p. To detect $\text{CD4}^{+}\text{CD39}^{+}$ Treg cells in rat models, $\text{CD4}^{+}\text{CD39}^{+}$ Tregs were obtained from Yubo Biotech (cat. no. YB-72865; <https://www.yubiotech.com>). For *in vitro* analysis, $\text{CD4}^{+}\text{CD39}^{+}$ Tregs were cultured with miR-27b-3p in triplicate wells of 6-well plates for 24 h. The cells were then transferred to flow cytometry tubes (grouped by experimental conditions) and incubated with fluorescein isothiocyanate-labeled monoclonal antibodies against CD4 (1:200; cat. no. YB70948; <https://www.yubiotech.com>) and CD39 (1:100; LM-6526R-FITC; LMAI Biotech) at 4°C for 30 min in the dark. After incubation, the cells were washed twice with PBS, fixed with 4% paraformaldehyde ($20\text{--}25^{\circ}\text{C}$), and analyzed by flow cytometry (FACS Aria II, BD Biosciences) to quantify $\text{CD4}^{+}\text{CD39}^{+}$ Tregs. For detecting $\text{CD4}^{+}\text{CD39}^{+}$ Treg cells in the NP tissue, the tissue samples were harvested from rat IVDD models. These tissue samples were processed and stained (4°C , 12-16 h) with monoclonal antibodies against CD4 and CD39 as aforementioned (4°C). After fixation with 4% paraformaldehyde for 15 min at room temperature ($20\text{--}25^{\circ}\text{C}$), flow cytometry was used to analyze the expression of CD4 and CD39, identifying and quantifying $\text{CD4}^{+}\text{CD39}^{+}$ Tregs in the NP tissue.

Table I. Primer sequences.

Primer name	Sequence (5'-3')
Rno-miR-27b-3p	F: CCGGATTCACAGTGGCTAAG R: AGTGCAGGGTCCGAGGTATT
Rno-miR-27b-3p reverse transcription	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACGCAGAA
Rat-U6	F: CTCGCTTCGGCAGCACA
Rat-U6	R: AACGCTTCACGAATTTGCGT

F, forward; R, reverse.

Animal research. Male Sprague-Dawley rats (body weight 22-250 g; obtained from Qinglongshan Zoo) were randomly assigned to one of six groups (n=6 per group; total 36 animals). The animals were ~10 weeks of age at the start of the experiment. Rats were housed in a specific pathogen-free (SPF) facility under controlled environmental conditions: temperature 22±2°C, relative humidity 50-60%, and a 12 h light/dark cycle (lights on at 07:00). Animals had free access to standard laboratory chow and water *ad libitum* throughout the study: the NC (Normal Control) group, PBS group, LPS (lipopolysaccharide) group, and LPS + miR-27b-3p group. The rats were anesthetized with 1% pentobarbital sodium (40 mg/kg) for the procedure. A 20 G needle was inserted into the lamina, and the injection site was held for 30 sec. The NP tissues of the rats in the experimental groups were injected with PBS, miR-27b-3p, or LPS at the Co5/6 and Co6/7 discs using a 22 G needle. The injections were repeated 4 weeks later to assess disc degeneration and treatment effects.

Numerous studies have used acupuncture of the coccygeal vertebra and LPS to generate rat models of disc degeneration. As compared with the NC group, LPS increased expression of MMP-13 in the disc degeneration model, thus creating a metabolic imbalance of the ECM to induce disc degeneration. The PBS treatment in this experiment did not exacerbate the degeneration of NP tissue. Instead, the PBS injection was used as a control in the context of the disc puncture model. The puncture itself induces disc degeneration, and the injection of PBS was intended to simulate the physical injury and maintain consistency in the experimental design. It was not expected to worsen the degeneration beyond the effect of the puncture. In addition, LPS also increased expression of caspase-3 and BMP-2 in IVD tissues, which accelerated inflammation-induced chondrocyte degeneration (33-35). The Co5/6 and Co6/7 discs were specifically selected for observation due to their anatomical relevance and accessibility in the rat cervical spine. These discs are frequently involved in IVDD in animal models and are critical for understanding the progression of cervical disc degeneration, which has translational relevance to human disc diseases. Moreover, the Co5/6 and Co6/7 discs are easily accessible in rats without excessive tissue damage, making them ideal for injection treatments. Previous studies have demonstrated that these discs provide consistent results in modeling IVDD, ensuring reliable and reproducible outcomes for studying the effects of miR-27b-3p on disc degeneration (36,37). The present study was conducted in accordance with the ARRIVE guidelines (38).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. To evaluate the differential expression of miR-27b-3p in the IVDD model, total RNA was isolated from blood samples collected from rats with disc degeneration and NCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. The concentration and purity of the total RNA was assessed with a spectrophotometer (ND-2000; NanoDrop Technologies LLC), and samples with an A260/280 ratio >1.8 were considered to have acceptable purity. Quality RNA was reverse transcribed into complementary DNA using a reverse transcription kit (Biosharp Life Sciences) with the primers listed in Table I, in accordance with the manufacturer's instructions. and qPCR was performed using a universal fluorescent quantitative PCR kit (Biosharp Life Sciences) on a [qPCR platform, for example, Applied Biosystems 7500 system]. Amplification was monitored with SYBR Green fluorophore. GAPDH was used as the internal reference gene. Thermocycling conditions were as follows: initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. A melting curve analysis was performed to confirm amplification specificity. Relative expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method (39), where C_q values were normalized to GAPDH. Due to the small size of the miRNA sequence, conventional primers are not effective for its amplification. To overcome this limitation, a stem-loop structure was introduced to elongate the miRNA sequence, which allowed us to design primers for efficient amplification. The reverse primer was designed based on this stem-loop structure, which is why the reverse primers for different miRNAs are consistent. The Rno-miR-27b-3p RT was specifically used to add the stem-loop sequence during reverse transcription, enabling accurate quantification of Rno-miR-27b-3p.

Magnetic resonance imaging (MRI). The rats were sacrificed at 8 weeks after spinal puncture by injection with sodium pentobarbital (200 mg/kg). The structure of the Co5/6 and Co6/7 discs was examined using a 3.0T MRI scanner (Bruker BioSpin GmbH) and analyzed with RadiAnt DICOM Viewer software (<https://www.radiantviewer.com/>). The extent of IVDD was assessed with the Pfirrmann grading scale.

Immunohistochemical analysis. Caudal vertebrae specimens from rats were fixed in 10% neutral-buffered formalin at room temperature (20-25°C) for 24-48 h, and subsequently

decalcified, dried, embedded in paraffin, and then sectioned at 4–5 μm . The paraffin-embedded sections were deparaffinized in xylene and rehydrated through a graded ethanol series (100, 95, 70%) to distilled water, stained with hematoxylin and eosin (H&E) and saffron O-fast green, dried, mounted on glass slides, and examined under a fluorescent microscope. Two of the authors evaluated the degree of IVDD in each group.

Single-cell transcriptomic analysis. The publicly available single-cell RNA sequencing dataset GSE205535 from normal (Normal_NP) and degenerated (Degenerate_NP) IVD samples (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE205535>) was analyzed. First, data quality control, normalization, and principal component analysis were performed using the Seurat package, followed by UMAP for dimensionality reduction to identify different cell populations within the samples. CD4⁺CD39⁺ Tregs were identified and clustered based on the CD4 and ENTPD1 (CD39) markers. Differential expression analysis (using the FindMarkers function) was conducted to compare CD4⁺CD39⁺ Tregs between normal and degenerated disc samples, identifying significantly differentially expressed genes. Gene Ontology (GO) enrichment analysis was performed to explore the functions of these genes. To further investigate the regulatory role of miR-27b-3p in CD4⁺CD39⁺ Tregs, GO enrichment analysis was performed using DAVID Bioinformatics Resources 6.8 (<https://david.ncifcrf.gov/>). To further investigate the regulatory role of miR-27b-3p in CD4⁺CD39⁺ Tregs, target genes were predicted using multiple databases, including TargetScanHuman 8.0 (https://www.targetscan.org/vert_80/), miRDB (<http://mirdb.org/>), and miRTarBase (<https://mirtarbase.cuhk.edu.cn/>). The expression of these predicted target genes was then compared between normal and degenerated samples. Finally, GO functional enrichment analysis and a volcano plot were used to visualize the differential expression of miR-27b-3p target genes, revealing the potential role of miR-27b-3p in regulating CD4⁺CD39⁺ Tregs.

Statistical analysis. All tests were conducted in triplicate or with a minimum of three distinct samples. The mean $\pm$ standard deviation was used to report continuous data in SPSS 22.0 program (IBM Corp.). An unpaired Student's t-test was used to compare the mean values of two independent groups. A one-way ANOVA followed by Tukey's post hoc test was conducted to compare the mean values across multiple independent groups, with statistically significant difference determined by $P < 0.05$. Statistical maps were generated using GraphPad Prism 8 software (Dotmatics).

Statistics and reproducibility. All experiments were repeated at least three times, and results are expressed as the mean $\pm$ standard error of the mean (SEM). Data analysis was performed using GraphPad Prism 9 and R language. Various statistical packages in R were utilized, including ggplot2 for data visualization, lme4 for linear mixed-effects model analysis, survival for survival analysis, and DESeq2 for differential gene expression analysis. Key experiments were independently repeated under different conditions, demonstrating consistent results to ensure reproducibility. Sample sizes were determined by power analysis to ensure adequate statistical power.

Results

Effects of miRNAs on IVDD. MR analyses were used to assess the causal relationship between 76 miRNAs and IVDD. As shown in Fig. 1A, only miR-27a-3p had a significant causal relationship with IVDD [odds ratio (OR)=1.12; 95% confidence interval (CI)=1.06–1.18; $P < 0.001$] (Table SII). Then, the leave-one-out method was used to separately remove the exposure of SNPs and the MR analysis was repeated. The overall results were not affected by a single SNP (Fig. 1B). A scatter plot generated to summarize the MR analysis results between miR-27a-3p and IVDD showed that the lines between different algorithms were generally upward, indicating that the risk of IVDD increased with the expression level of miR-27a-3p (Fig. 1C). The funnel plot results eliminated possible outliers and the opportunity for miR-27a-3p levels to be multidirectional (Fig. 1D).

Effects of immune cell on IVDD. A total of five MR analysis modalities were employed to identify potential relationships between immune cells and IVDD. The findings indicated that 35 types of immune cells were significantly associated with IVDD ($P < 0.05$ with the IVW technique) (Fig. 2A; Table SIII). Moreover, 19 types of immune cells were positively correlated to IVDD when the value was ≤ 0 , whereas 16 types of immune cells were positively correlated to IVDD when the value was > 1 (Fig. 2B). Then, in order to exclude false positive results, FDR correction was performed on the MR results obtained from the batch analysis. The results identified that 28 types of immune cells were causally related to IVDD (Table SIV).

CD4 on CD4⁺CD39⁺ Treg cells is positively correlated with miR-27a-3p. Two-sample MR analysis was conducted with 35 types of immune cells as outcome factors and miR-27a-3p as the exposure factor to assess the relationship between miR-27a-3p and IVDD. The results revealed a significant causal relationship between miR-27a-3p and CD4 on CD4⁺CD39⁺ Tregs (OR=1.17, 95% CI=1.03–1.34, $P = 0.02$) (Fig. 3A) (Table SV). The leave-one-out results revealed no bias effect of individual SNPs on the overall results (Fig. 3B). The scatter plot results showed an overall negative correlation of miR-27a-3p and CD4 on CD4⁺CD39⁺ Tregs (Fig. 3C). The funnel plot (Fig. 3D) excluded any potential outliers and the possibility of multi-directivity of miR-27a-3p levels. Reverse MR analysis, which was conducted to improve the reliability of the results, found no reverse causality of miR-27a-3p and CD4 on CD4⁺CD39⁺ Tregs.

Effect of miR-27a-3p on IVDD was achieved by regulating CD4 on CD4⁺CD39⁺ Tregs. Multiple MR analyses confirmed a causal relationship between IVDD and CD4 on CD4⁺CD39⁺ Tregs (OR=1.03; 95% CI=1.004–1.060; $P = 0.024$) (Fig. 4A). Analysis with the leave-one-out method showed no deviation from the overall result of each SNP (Fig. 4B). The scatter plot results demonstrated that the lines of the five algorithms generally tilted upward, indicating a positive correlation between CD4⁺CD39⁺ Treg cells and IVDD (Fig. 4C). The funnel plot (Fig. 4D) excluded possible outliers and opportunities for multi-directionality of CD4⁺CD39⁺ Treg cell levels. In addition, reverse MR analysis to improve the reliability

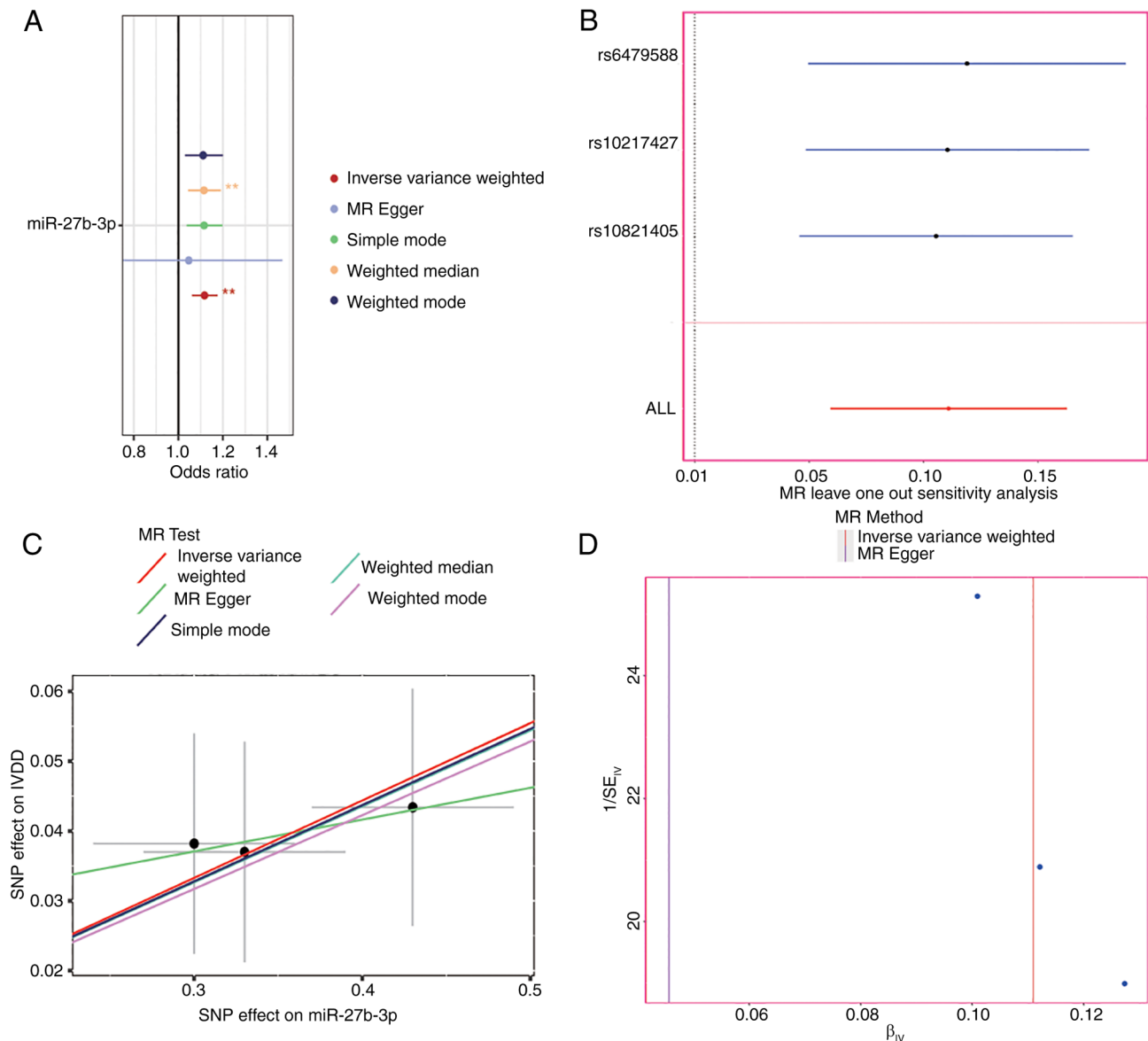


Figure 1. MR analysis of the effect of miR-27a-3p on IVDD. (A) A forest map of 5 MR analysis methods. (B) MR analysis of miR-27a-3p and IVDD with the leave-one-out approach. (C) Scatter plots of the genetic association between miR-27a-3p and risk of IVDD. (D) Funnel plots of the instrumental variables for each significant causal relationship between miR-27a-3p and IVDD. MR, Mendelian randomization; miR, microRNA; IVDD, intervertebral disc degeneration.

of the results found no reverse causality between IVDD and CD4⁺CD39⁺ Tregs.

MiR-27a-3p enhances LPS-induced apoptosis and modulates ECM markers in NP cells. Additional experiments were conducted using LPS, miR-27a-3p, miR-27a-3p inhibition, LPS + miR-27a-3p and LPS + miR-27a-3p inhibition to investigate the function of miR-27a-3p in IVDD. The results of the TUNEL assay indicated that both LPS and miR-27a-3p promoted apoptosis of NP cells as compared with the NC group. In addition, miR-27a-3p enhanced LPS-induced apoptosis of NP cells. The inclusion of the miR-27a-3p inhibitor and LPS + miR-27a-3p inhibitor groups further confirmed the involvement of miR-27a-3p in mediating apoptosis in NP cells (Fig. 5A). The results of western blot analysis indicated that LPS and miR-27a-3p lowered the expression levels of aggrecan and COL2A in NP cells. Expression of the apoptosis-related

protein Bax was increased. Furthermore, miR-27a-3p enhanced the effect of LPS on the expression levels of these proteins (Fig. 5B-E). Suppression of miR-27a-3p activity prevented the decrease in aggrecan and COL2A expression in LPS-treated NP cells, while decreasing expression of the apoptosis-related protein Bax (Fig. 5F-I). Although BAX is directly related to the regulation of NP cell apoptosis, aggrecan and COL2A are usually used as markers for assessing IVDD, mainly for evaluating the degradation of ECM and the integrity of the IVD. Aggrecan and COL2A were selected to assess the degenerative changes of NP cells, which is a key feature of IVDD, while BAX and TUNEL focus on apoptosis.

Increasing the proportion of CD4⁺CD39⁺ Tregs by miR-27a-3p promotes apoptosis of NP cells. The addition of miR-27b-3p significantly increased the apoptotic rate of CD4⁺CD39⁺ Tregs as compared with the NC group (Fig. 6A and B). CD4⁺CD39⁺

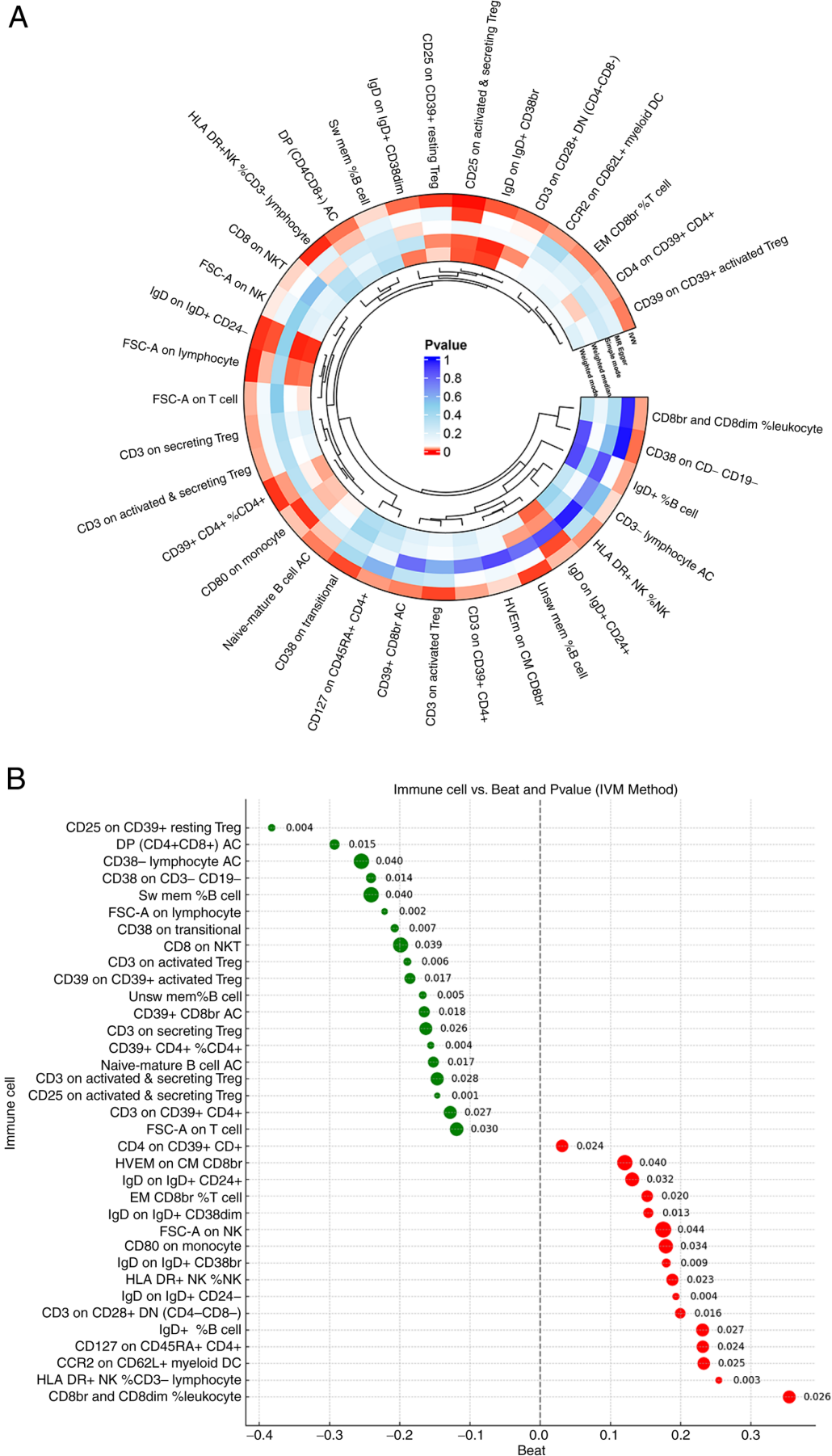


Figure 2. Correlation and association analysis of immune cells and their relationship with IVDD. (A) Heatmap showing the P-values of the association between different immune cell types and various parameters. (B) Forest plot depicting the correlation between immune cell types and key variables related to IVDD using the IVW method. IVDD, intervertebral disc degeneration; IVW, Inverse variance weighting.

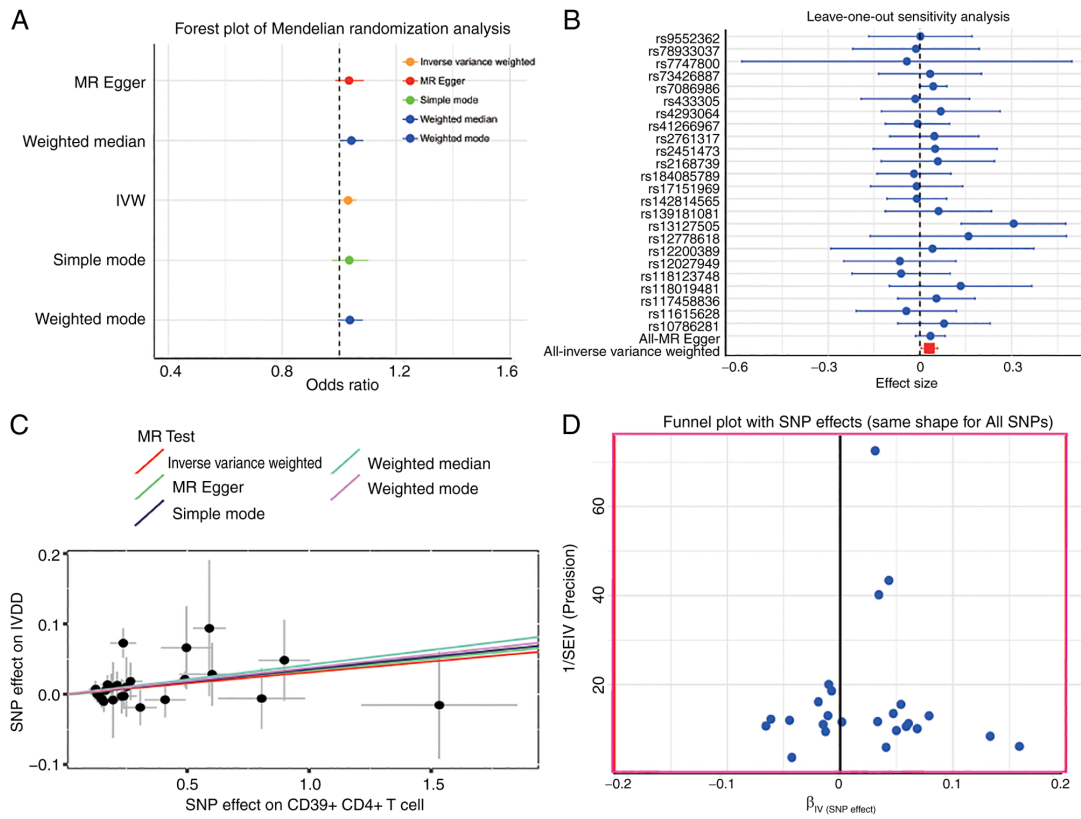


Figure 3. MR analysis of the effect of miR-27a-3p on CD4 of CD4⁺CD39⁺ Tregs. (A) Forest map of five MR analysis methods. (B) MR analysis of the causal relationship between miR-27a-3p and CD4 on CD4⁺CD39⁺ Tregs with the leave-one-out method. (C) Scatter plots showing genetic association between miR-27a-3p and CD4 on CD4⁺CD39⁺ Treg cells. (D) Funnel plots of the instrumental variables for each significant causal relationship between miR-27a-3p and CD4 on CD4⁺CD39⁺ Treg cells. MR, Mendelian randomization; miR, microRNA; Tregs, regulatory T cells; SNPs, single nucleotide polymorphisms.

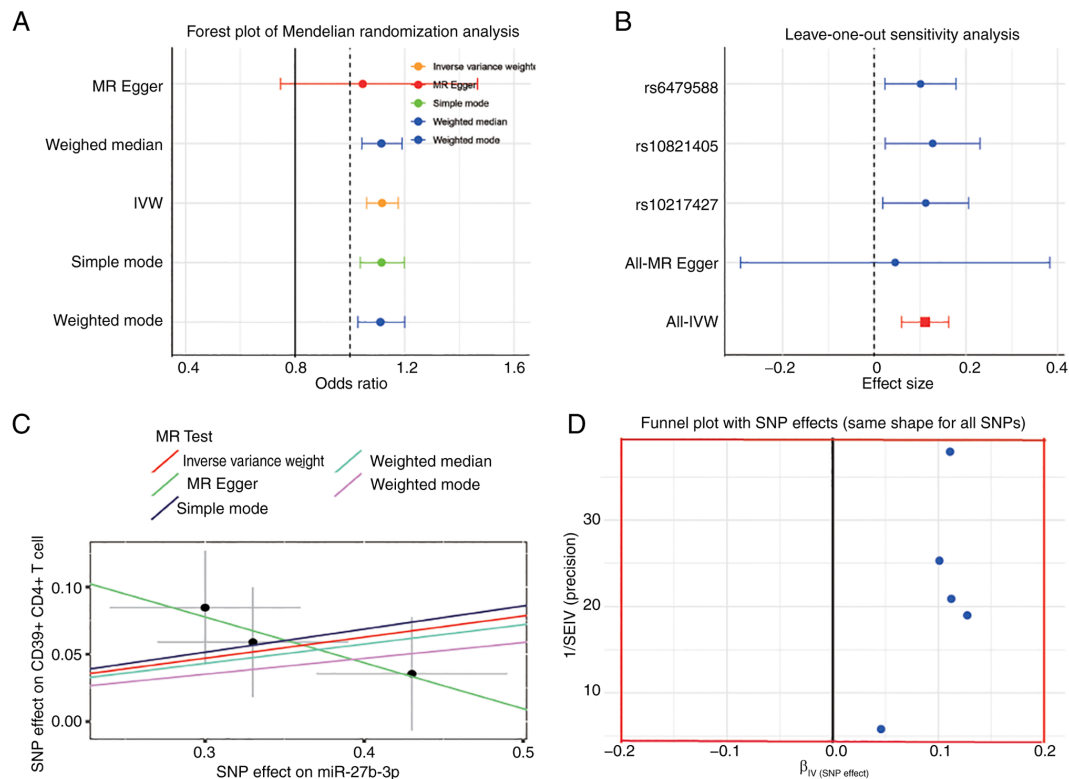


Figure 4. MR analysis of the effect of miR-27a-3p on CD4 on CD4⁺CD39⁺ Tregs. (A) Forest map of five MR analysis methods. (B) MR analysis of miR-27a-3p and CD4 on CD4⁺CD39⁺ Tregs with the leave-one-out method. (C) Scatter plots showing the genetic association between miR-27a-3p and CD4 on CD39⁺CD4⁺ Tregs. (D) Funnel plots of the instrumental variables for each significant causal relationship between miR-27a-3p and CD4 on CD4⁺CD39⁺ Tregs. MR, Mendelian randomization; miR, microRNA; Tregs, regulatory T cells; IVW, Inverse variance weighting; SNPs, single nucleotide polymorphisms.

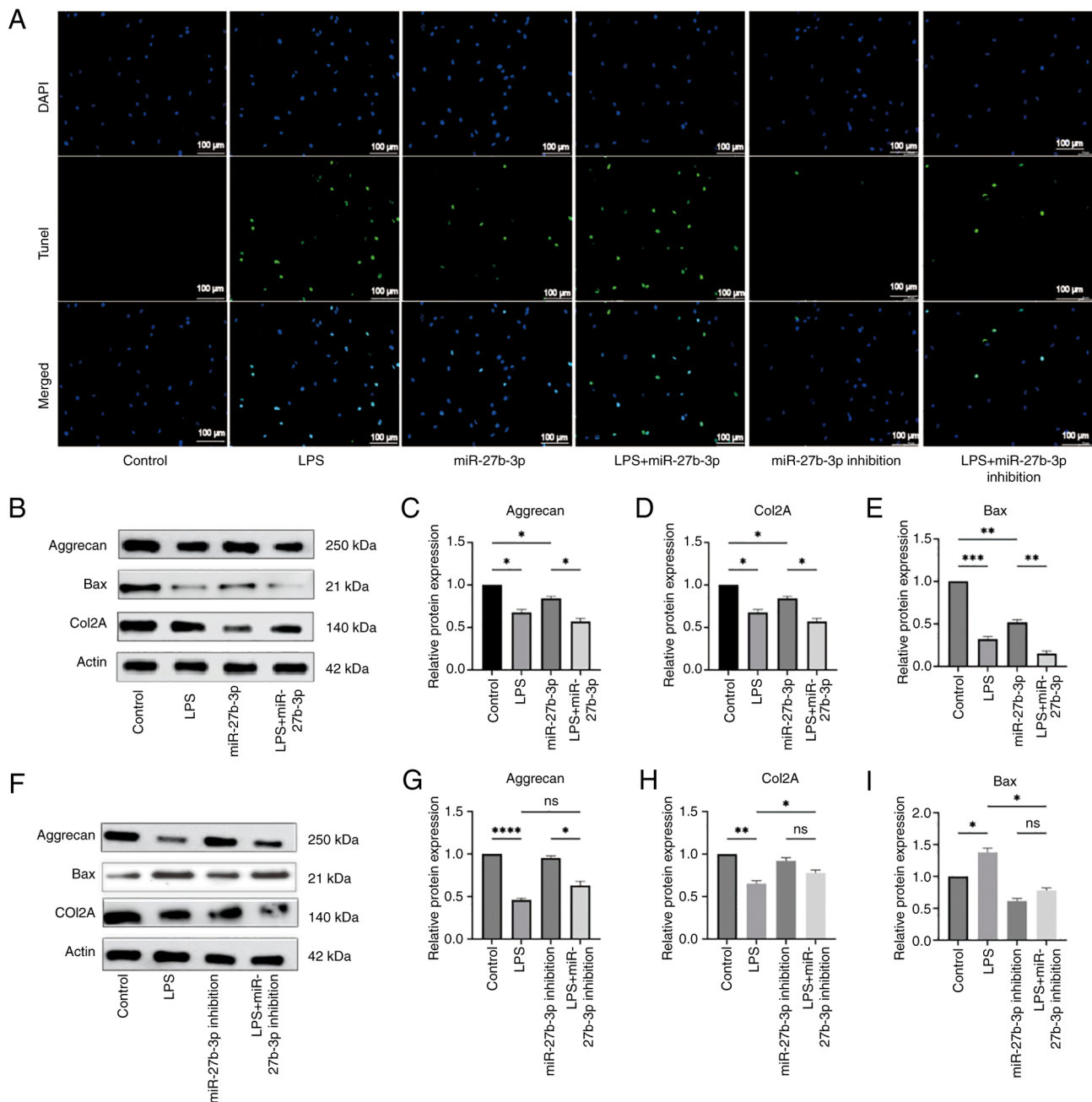


Figure 5. Apoptosis of NP cells is induced by miR-27a-3p. (A) TUNEL analysis of cells in the NC, LPS, miR-27a-3p, LPS + miR-27a-3p, miR-27a-3p, inhibition, and LPS + miR-27a-3p inhibition groups. (B-E) Expression levels of apoptosis-related proteins and IVDD-related proteins of cells in the NC, LPS, miR-27a-3p, and LPS + miR-27a-3p groups. (F-I) Expression levels of apoptosis-related proteins and IVDD-related proteins of cells in the NC, LPS, miR-27a-3p inhibition and LPS + miR-27a-3p inhibition groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. NP, nucleus pulposus; miR, microRNA; NC, negative control; LPS, lipopolysaccharide; IVDD, intervertebral disc degeneration; ns, not significant.

Tregs and miR-27b-3p combined increased the proportion of apoptotic NP cells (Fig. 6C and D). The expression of CD4⁺CD39⁺ Tregs in blood was increased in rats with more severe disc degeneration (Fig. 6E). In rats with more severe disc degeneration, the expression of CD4⁺CD39⁺ Tregs in the NP of the IVD was increased (Fig. 6F).

Progression of IVDD is accelerated by miR-27a-3p. Histological analysis was conducted of the caudal vertebra of rats following injection of PBS, LPS, and LPS + miR-27a-3p. Staining with H&E and carthamin fast green (0.5%; MilliporeSigma) revealed that the disc shape of the NC group

was complete and the NP and annulus fibrosus (AF) tissues were neatly arranged. Injection of PBS and LPS altered the integrity of the discs, resulting in a reduction in NP tissue. As compared with the LPS group, LPS + miR-27a-3p reduced IVD integrity and the amount of NP tissue (Fig. 7A and C). MRI of the degenerated discs showed structural variability, changes in signal strength, and blurred distinction between NP and AF tissues. MRI also showed that injection of PBS and LPS promoted disc degeneration as compared with the NC group. Moreover, disc degeneration was more severe in the LPS + miR-27a-3p group as compared with the LPS group (Fig. 7B and D). Comparisons of the blood parameters

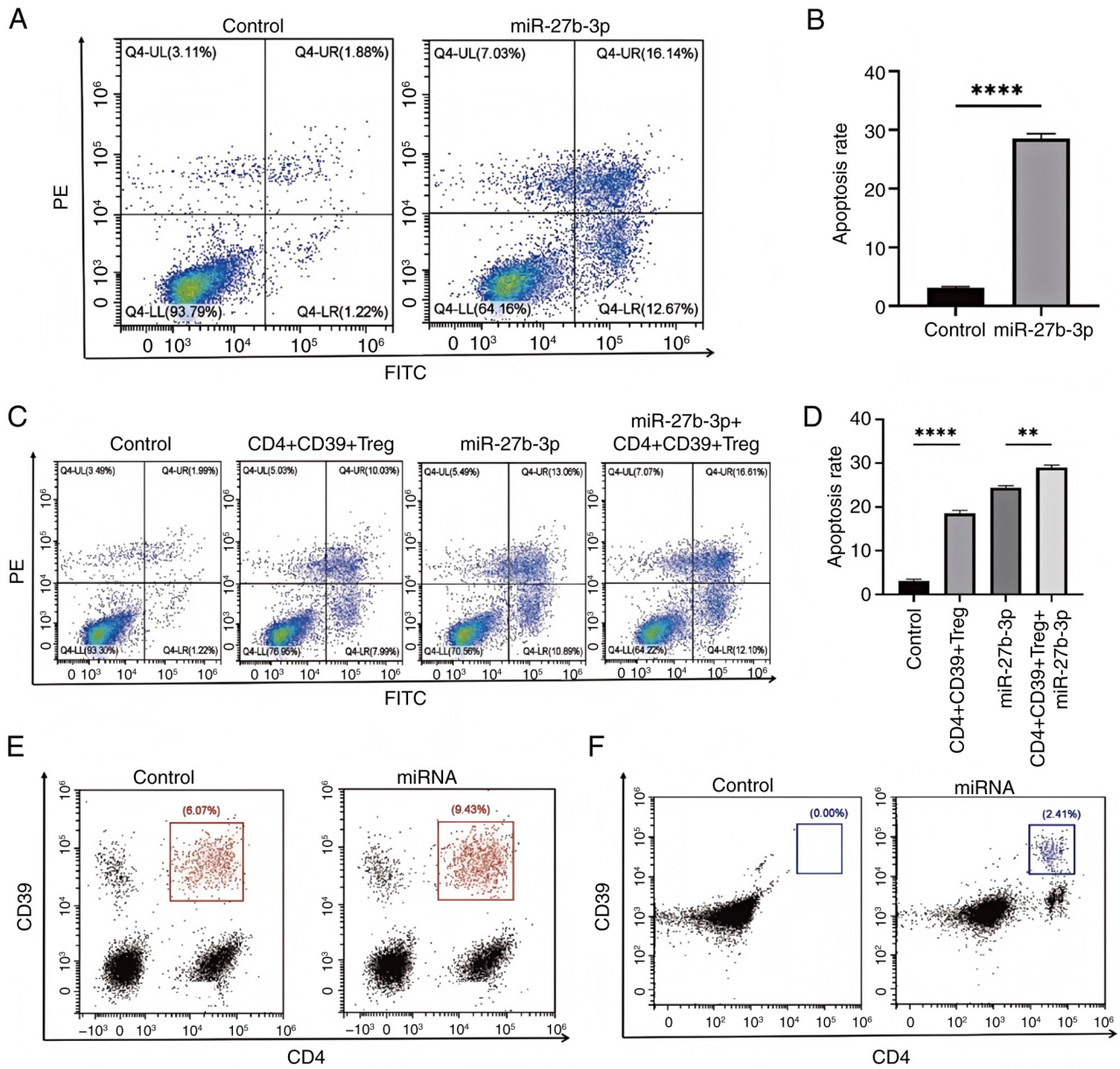


Figure 6. miR-27a-3p promotes NP cell apoptosis through CD4⁺CD39⁺ Tregs. (A) Flow cytometry of apoptosis in CD4⁺CD39⁺ Tregs after addition of miR-27b-3p. (B) two groups of CD4⁺CD39⁺ Tregs' apoptosis rate of bar chart. (C) Flow cytometry of apoptosis of NP cells after adding miR-27b-3p, CD4⁺CD39⁺ Tregs and miR-27b-3p + CD4⁺CD39⁺ Tregs. (D) Bar chart demonstrating the apoptotic rate of NP cells. (E) Flow cytometry of blood CD4⁺CD39⁺ Tregs from normal and IVDD rats. (F) Flow cytometry of CD4⁺CD39⁺ Tregs in NP tissues from normal rats and IVDD rats. **P<0.01 and ****P<0.0001. miRNA or miR, microRNA; NP, nucleus pulposus; Tregs, regulatory T cells.

of normal rats and rats with IVDD by RT-qPCR analysis demonstrated that serum levels of miR-27a-3p were higher in IVDD rats than the control rats (Fig. 7E). In the NC group, Aggrecan and Col2A are highly expressed with intact disc structure. The PBS group (Control) showed a slight reduction in fluorescence, indicating mild degeneration. The LPS group (Degeneration Model) had significantly lower expression, with weakened signals and clear signs of degeneration. In the LPS + miR-27b-3p group (Degeneration with Treatment), further reduced fluorescence suggests the treatment worsens disc degeneration (Fig. 7F-H). To further verify the role of CD4⁺CD39⁺ Tregs in IVDD, the IF staining data of the PBS group and the LPS + miR-27b-3p group were supplemented. The results identified a significant increase in CD4⁺CD39⁺ Tregs in the LPS + miR-27b-3p group, further supporting that

miR-27b-3p promotes the immune-mediated process of IVDD by regulating immune cells, especially CD4⁺CD39⁺ Tregs (Fig. S2).

Single-cell transcriptomic analysis reveals the regulatory role of miR-27b-3p in CD4⁺CD39⁺ Tregs during IVDD. In the present study, CD4⁺CD39⁺ Tregs from normal and degenerated IVD samples were analyzed using the single-cell transcriptomic dataset GSE205535. The UMAP dimensionality reduction (Fig. 8A and B) revealed notable differences in the distribution and clustering of CD4⁺CD39⁺ Tregs between the Normal_NP and Degenerate_NP disc samples. Specifically, the Normal_NP samples exhibited a more homogeneous distribution of CD4⁺CD39⁺ Tregs, while the Degenerate_NP samples showed a more scattered distribution, suggesting a

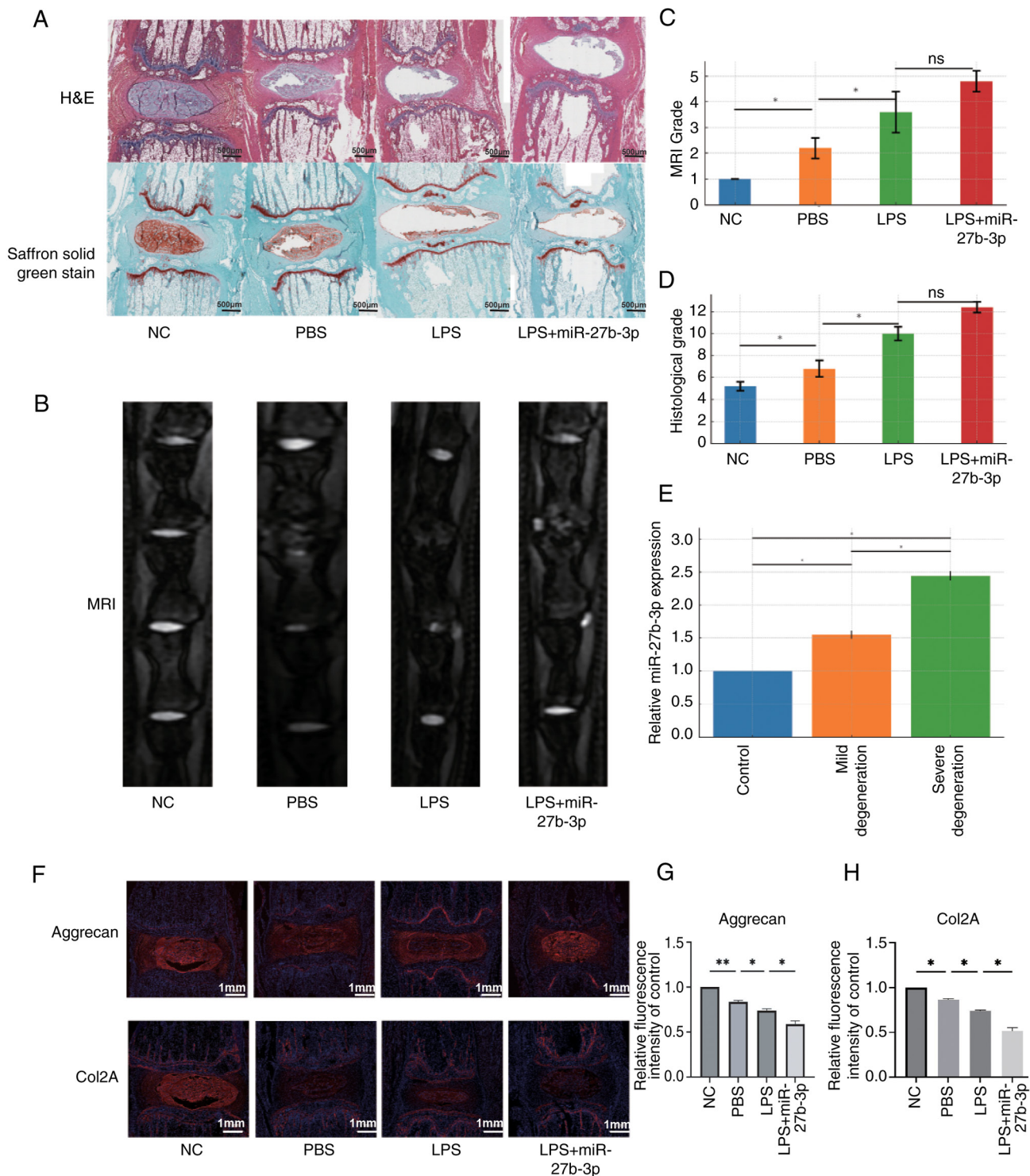


Figure 7. Intervertebral disc degeneration in rats is promoted by miR-27a-3p. (A) H&E and saffron green staining of each group. (B) MRI of the tail vertebral body of rats after relevant treatment. (C) Degeneration grading of the tail vertebral disc of rats. (D) Histological grading of degeneration of the rat tail vertebral disc. (E) Degeneration grading of the tail vertebral disc of rats. (F) Fluorescence staining of Aggrecan and Col2A in intervertebral disc tissue across different groups. Red indicates positive signals, while blue represents background. (G and H) Immunofluorescence intensity of Aggrecan and COL2A was quantified, showing significant changes in expression levels between groups. * $P < 0.05$ and ** $P < 0.01$ ($n = 3$). miR, microRNA; MRI, magnetic resonance imaging; NC, negative control; LPS, lipopolysaccharide.

shift in the composition of these immune cells in the context of disc degeneration (Fig. 8C and D). These findings highlight the altered immune landscape in degenerated discs, with potential implications for immune-mediated processes in IVDD. Differential expression analysis identified significantly altered genes in $CD4^+CD39^+$ Tregs in the degenerated samples

compared with normal samples, particularly genes related to apoptosis and immune regulation (Fig. 8E and F). GO enrichment analysis further showed that these differentially expressed genes are involved in key immune-related biological processes such as monocyte activation and zinc ion response (Fig. 8F). Trajectory analysis using pseudotime ordering

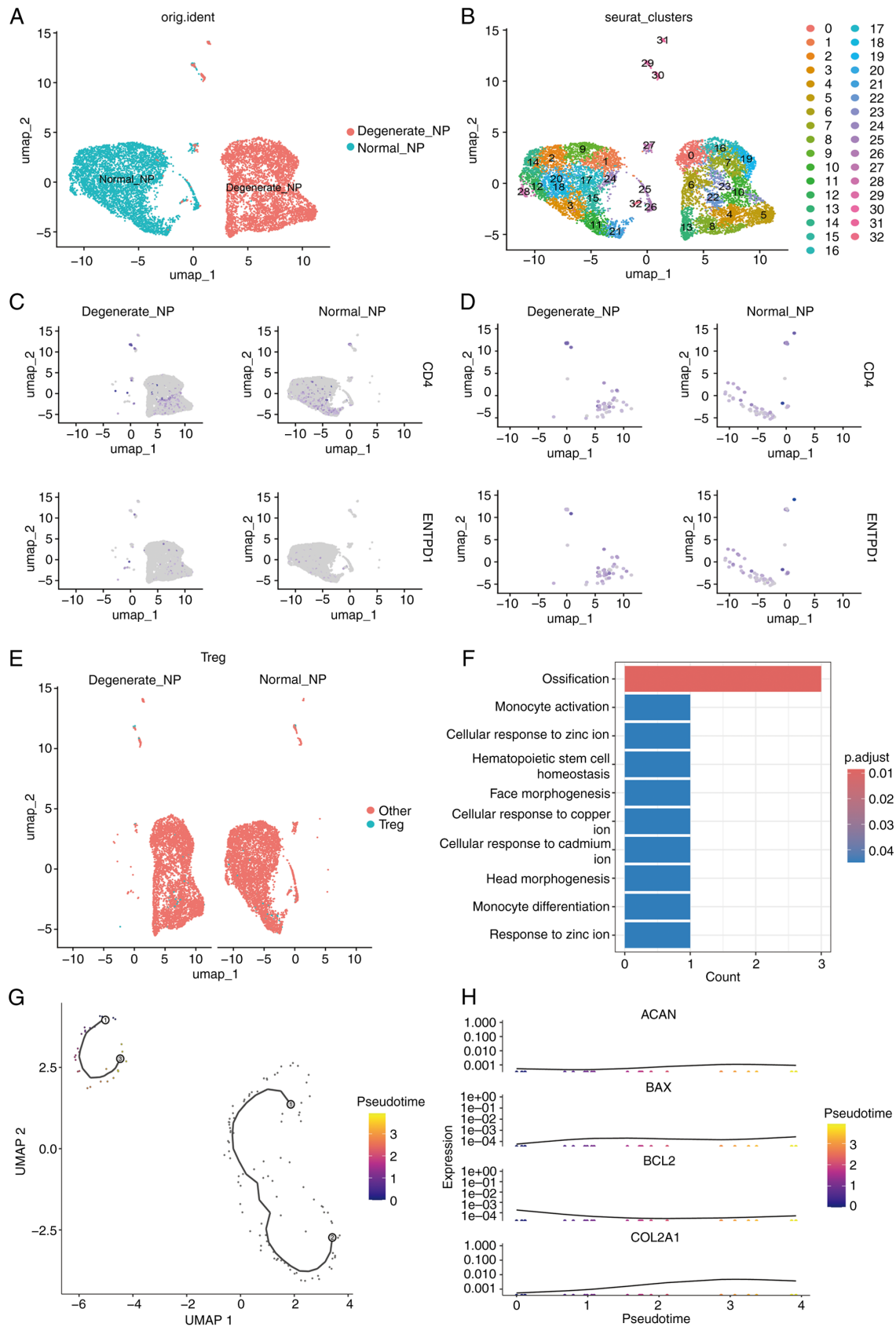


Figure 8. UMAP and functional analysis of CD4⁺CD39⁺ Tregs in normal and degenerated intervertebral discs. (A and B) UMAP plots showing the distribution of CD4⁺CD39⁺ Tregs in Normal_NP and Degenerate_NP intervertebral disc samples. The cells are clustered according to their gene expression profiles, revealing distinct populations in each condition. Normal_NP samples exhibit a more compact and uniform distribution of Tregs, while Degenerate_NP samples show a more dispersed distribution, suggesting a shift in Treg cell composition during disc degeneration. (C and D) Feature plots highlight the expression of CD4 and ENTPD1 (CD39), used to identify Tregs. (E) Distribution of CD4⁺CD39⁺ Tregs across different conditions. (F) Results of Gene Ontology enrichment analysis, showing significant involvement of these cells in biological processes such as monocyte activation and zinc ion response. (G) Pseudotime trajectory analysis demonstrating dynamic state transitions of CD4⁺CD39⁺ Tregs. (H) Pseudotime-associated gene expression trends of *ACAN*, *BAX*, *BCL2*, and *COL2A1*, illustrating altered regulation of extracellular matrix and apoptosis pathways during degeneration. Tregs, regulatory T cells; NP, nucleus pulposus.

(Fig. 8G) revealed a dynamic progression of CD4⁺CD39⁺ Tregs from normal to degenerated samples, suggesting state transitions associated with disc pathology. Furthermore, pseudotime-associated expression patterns (Fig. 8H) showed that key genes, including ACAN, BAX, BCL2, and COL2A1, exhibited distinct trends across the trajectory, highlighting altered regulation of extracellular matrix homeostasis and apoptosis-related pathways during degeneration. Additionally, bioinformatics tools were used to predict the target genes of miR-27b-3p, and distinct expression patterns of these target genes were found in CD4⁺CD39⁺ Tregs (Fig. 9A). Notably, genes such as PPARG, WEE1 and MMP13 were significantly upregulated or downregulated in degenerated discs (Fig. 9B). GO functional enrichment analysis indicated that these target genes are closely associated with processes such as immune cell migration, regulation of immune responses and cholesterol metabolism (Fig. 9C). The volcano plot further revealed the differential expression patterns of these target genes (Fig. 9D), suggesting that miR-27b-3p may exert its role in IVDD by regulating CD4⁺CD39⁺ Tregs and their associated immune functions.

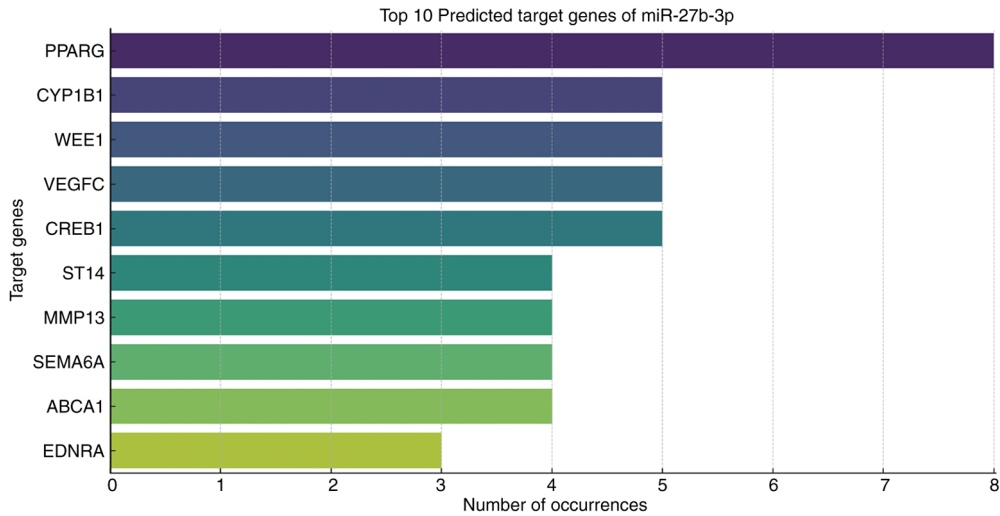
Discussion

Our study breaks new ground by demonstrating the causal role of miR-27b-3p in IVDD through MR, a method rarely applied in this context. This approach minimizes confounding factors and strengthens the evidence for miR-27b-3p as a potential therapeutic target. Additionally, the discovery of its interaction with CD4⁺CD39⁺ Treg cells adds a novel dimension to our understanding of immune-related degeneration processes, making this research highly relevant to both the fields of immunology and aging. MR analysis and experimental validation both *in vitro* and *in vivo* yielded compelling evidence on the association between miR-27a-3p and IVDD. Two-sample MR analysis of 76 miRNAs and IVDD revealed a significant causal association between miR-27a-3p and IVDD. A total of 731 immune cell types were incorporated as mediators to gain a deeper understanding of potential causal links. MR analysis of 731 immune cell types and IVDD identified 35 immune cell types causally linked to IVDD. Two-sample MR analysis was conducted using immune cells as the dependent variable and miR-27a-3p as the instrumental variable. The MR analysis using human samples revealed a significant causal relationship between miR-27b-3p and IVDD. These results suggest that miR-27b-3p could serve as a viable biomarker for early diagnosis or as a target for therapeutic intervention in clinical settings. The findings indicate that miR-27b-3p plays a crucial role in the progression of IVDD by inducing NP cell apoptosis, a key feature of disc degeneration. This effect was demonstrated both *in vitro* and *in vivo*. Additionally, miR-27b-3p modulates the immune response by influencing CD4⁺CD39⁺ Treg cells, which are important regulators of inflammation and immune responses. It was observed that miR-27b-3p affects apoptosis-related proteins, such as Bax, promoting NP cell apoptosis, while simultaneously impacting the expression of ECM components such as COL2A and aggrecan, which are critical for maintaining disc structure and function. The use of LPS in IVDD models was crucial for inducing inflammation, providing a platform to study the

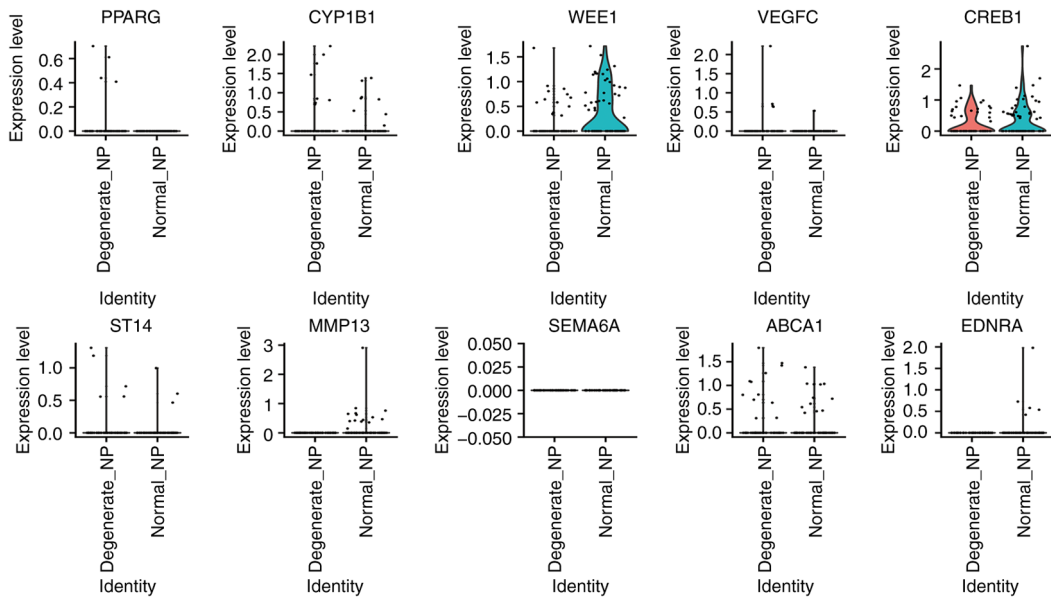
inflammatory component of IVDD. miR-27b-3p was found to enhance the LPS-induced inflammatory response, further exacerbating disc degeneration by promoting apoptosis and matrix degradation. In addition, the current findings revealed that miR-27b-3p regulates several key genes involved in inflammation and matrix remodeling. PPARG, a nuclear receptor involved in immune regulation, was found to be downregulated by miR-27b-3p, suggesting that miR-27b-3p may contribute to inflammatory responses by interfering with PPARG signaling (40). Similarly, miR-27b-3p also modulates WEE1, a cell cycle regulator, which could impact the survival of NP cells by regulating cell cycle progression and response to DNA damage. Finally, miR-27b-3p was shown to regulate MMP13, a matrix metalloproteinase responsible for collagen degradation, contributing to the breakdown of the ECM in degenerated discs. The interaction of miR-27b-3p with these key regulatory pathways further supports its role as a central modulator in the pathogenesis of IVDD (41). This novel connection between miR-27b-3p and CD4⁺CD39⁺ Tregs highlights a previously unexplored pathway, where miR-27b-3p not only contributes to the apoptosis of NP cells but also regulates immune-mediated degeneration processes. These findings open new avenues for targeting miR-27b-3p in the treatment of IVDD, offering potential strategies for both immune modulation and preservation of disc integrity. It was chosen to focus on CD4⁺CD39⁺ Tregs in relation to IVDD primarily due to their role as intermediaries in the miR-27b-3p-mediated regulation of IVDD. Our MR analysis examined 731 immune cell types and their potential causal relationships with IVDD. Among these, CD4⁺CD39⁺ Tregs were identified as significantly associated with the progression of IVDD. However, it is important to note that the focus on these cells was not due to their direct association with IVDD, but rather because they serve as key regulators of the immune response in the disc, specifically through their interaction with miR-27b-3p. It was hypothesized that miR-27b-3p influences the function of CD4⁺CD39⁺ Tregs, and in doing so, regulates NP cell apoptosis and ECM degradation during IVDD progression. Our MR analysis highlighted these cells as critical intermediaries in the pathway through which miR-27b-3p affects disc degeneration (42).

Recent studies have implicated miRNAs in several signaling pathways as significant contributors to the development of various illnesses (43,44). These tiny non-coding RNAs have the ability to inhibit translation, leading to reduced expression levels of certain genes (45). IVDD is a significant contributor to low back pain (46,47). Current treatments help to alleviate symptoms but are not curative. It is increasingly recognized that the dysregulation of miRNAs plays a crucial role in the progression of IVDD (48). Several miRNAs have been shown to regulate key processes involved in IVDD, such as inflammation, matrix degradation and apoptosis, making them promising therapeutic targets. For example, previous studies have identified miR-21 and miR-133a as key miRNAs involved in the degeneration of IVDs. miR-21 has been reported to contribute to IVDD by regulating the inflammatory response and promoting matrix degradation (49). Similarly, miR-133a has been shown to regulate ECM remodeling, and its downregulation is associated with increased disc degeneration (50). These findings highlight the importance of miRNAs

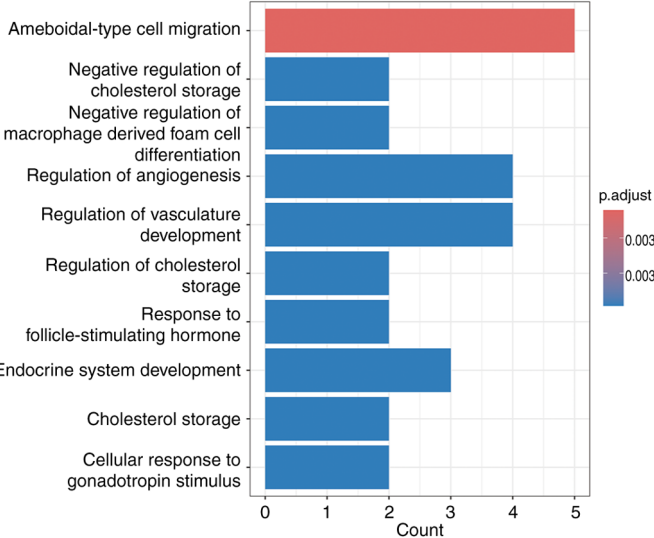
A



B



C



D

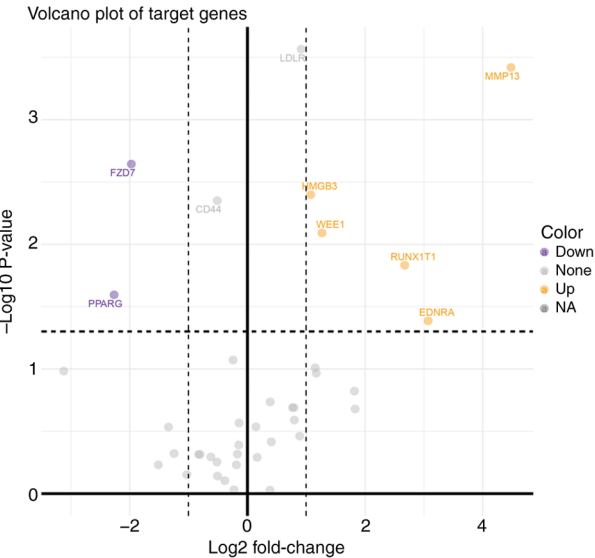


Figure 9. miR-27b-3p target gene analysis and functional impact on CD4⁺CD39⁺ regulatory T cells. (A) Predicted target genes of miR-27b-3p, including genes such as PPARG, CYP1B1 and WEE1. (B) Differential expression of these target genes in normal and degenerated intervertebral disc samples, with notable changes in genes such as PPARG, WEE1 and MMP13. (C) Gene Ontology functional enrichment analysis of miR-27b-3p target genes, highlighting their involvement in immune cell migration, immune response regulation and cholesterol metabolism. (D) Volcano plot of the differentially expressed miR-27b-3p target genes, revealing both upregulated and downregulated genes in degenerated discs. miR, microRNA.

in IVDD, yet the exact mechanisms by which they mediate disc degeneration remain under explored. In contrast to these studies, the present study focuses on miR-27b-3p, a member of the miR-27 family that has not been extensively studied in the context of IVDD (51). miR-27b-3p has been implicated in regulating immune responses and apoptosis, processes known to contribute significantly to IVDD progression. The current findings suggest that miR-27b-3p directly modulates CD4⁺CD39⁺ Tregs, which are key players in immune regulation and inflammation. It was observed that miR-27b-3p promotes NP cell apoptosis and matrix degradation by influencing the immune microenvironment in degenerated discs. This provides a novel insight into the immune-modulatory role of miR-27b-3p in IVDD, distinguishing our study from previous research that has mainly focused on other miRNAs like miR-21 and miR-133a. While previous studies have demonstrated the dysregulation of miR-21 and miR-133a in IVDD, the present study adds a new dimension by identifying miR-27b-3p as a key regulator of immune cells, specifically CD4⁺CD39⁺ Tregs, in the context of IVDD. Several studies have indicated that miR-27a plays a crucial role in modulation of polymorphisms, carcinogenesis, proliferation, apoptosis, invasion, migration, and angiogenesis (52). A recent study revealed that expression of miR-27a-5p was increased in the exosomes of mice as models of Alzheimer's disease (53).

CD4 T cells are crucial in facilitating adaptive immunity against several infections and have also been implicated in autoimmunity, asthma, allergic responses and tumor immunity (54,55). Naive CD4 T cells can develop into diverse T helper (Th) cell lineages, such as Th1, Th2, Th17 and induced Tregs, via TCR activation in particular cytokine environments. These lineages are characterized by cytokine production and specific functions (56,57). Treg and Th17 cells are two subgroups of CD4⁺ T cells with opposing effects. Th17 cells contribute to inflammation, whereas Tregs play a vital role in preserving immunological balance (58,59). Altered activity of Tregs plays a crucial role in the onset and progression of immuno-senescence, leading to greater vulnerability to age-related immune-mediated illnesses (60). The IVD is considered an immune privileged organ due to the lack of blood vessels. During IVDD, immune cells and inflammatory factors can enter the IVD by penetrating the cartilage endplate and AF tissues, leading to the development of IVDD. In recent decades, there has been an increasing number of publications on the inflammatory response in the IVD, which has garnered increasing interest in a causal link between CD4 Tregs and IVDD, highlighting prospective treatment approaches (61,62). CD4⁺ Tregs exert their regulatory effects by producing anti-inflammatory cytokines such as IL-10 and TGF- β , which inhibit the activation of pro-inflammatory T cells and macrophages. In the context of IVDD, the presence of Tregs in the IVD may help control the inflammatory cascade, reduce matrix degradation, and potentially promote tissue repair. Importantly, miR-27b-3p, as identified in the present study, may enhance Treg cell activity by regulating immune signaling pathways, further supporting its potential therapeutic value in modulating immune responses in IVDD. MR analysis strengthened the reliability of the findings of the first part of the present study. The consistency of five MR analysis modalities (weighted

median, MR-Egger, simple mode, weighted mode and IVW) enhanced the validity of the results (63). The MR-PRESSO approach was utilized to re-evaluate the presence of diverse SNPs, while Cochran's Q test was employed to evaluate the notable heterogeneity among the chosen instrumental factors (64,65). The miRNA identified by MR analysis was utilized in the IVDD model in conjunction with *in vitro* and *in vivo* investigations. The advantage of utilizing MR analysis is the use of existing data from large-scale genome-wide association studies, which can reveal potential causal relationships between modifiable factors and disease, while allowing the inclusion of risk factors with suitable genetic variants. Genetic variants typically have relatively small effects on most risk factors and explain only a small portion of the variation, while decreasing the statistical power of MR analyses and the risk of false-negative results. To avoid the risk of false-positive results, *in vivo* and *in vitro* experiments were designed based on the MR findings, thereby completing an evidence triangle and providing new research ideas for the prevention and treatment of IVDD.

There were certain limitations to the present study that should be addressed. First, the data were derived exclusively from Europeans. Second, although both *in vitro* and *in vivo* investigations demonstrated that miR-27a-3p can enhance the development of IVDD by promoting apoptosis of NP cells, the role CD4⁺CD39⁺ Treg cells in this process remains unclear. Finally, a miR-27b-3p alone treatment group was not included because our primary objective was to assess the role of miR-27b-3p in the context of LPS-induced inflammation, which is a key aspect of IVDD. LPS was used to model the inflammatory environment commonly associated with IVDD, and the aim was to observe the interaction between miR-27b-3p and inflammation. Although a miR-27b-3p alone group was not included, the influence of miR-27b-3p on NP cell apoptosis in the LPS + miR-27b-3p group was determined based on the significant changes observed in apoptosis-related markers (Bax) and ECM proteins (Aggrecan and COL2A). The fact that miR-27b-3p enhanced LPS-induced apoptosis suggests that miR-27b-3p plays an important role in regulating apoptosis in an inflammatory environment. However, it is acknowledged that a miR-27b-3p alone group would have provided additional clarity regarding its independent effect. Future studies could include such a group to improve understanding of miR-27b-3p's standalone effects.

The findings of the present study not only advance our understanding of miR-27b-3p's role in IVDD but also contribute to the broader understanding of aging-related degeneration. The modulation of CD4⁺CD39⁺ Tregs by miR-27b-3p suggests a potential mechanism through which immune aging accelerates musculoskeletal decline. These results align with emerging theories of 'inflammaging', where chronic low-grade inflammation contributes to age-related tissue degeneration. Targeting miR-27b-3p may, therefore, represent a novel therapeutic strategy to slow the progression of IVDD and possibly other aging-associated disorders. Future research could further explore the role of miR-27a-3p in other degenerative diseases, especially those involving immune system regulation. Given the high prevalence of IVDD and its significant impact on quality of life, the results

of the present study are expected to drive the development of new diagnostic biomarkers and offer novel therapeutic targets for personalized treatment strategies. By targeting miR-27a-3p or the immune cells it regulates, more effective therapies could be developed to slow down or even reverse the progression of IVDD, which holds broad potential for clinical applications. Additionally, future studies could investigate the generalizability of miR-27a-3p-related pathways in other inflammation-related degenerative diseases, paving the way for innovative cross-disease therapeutic strategies. Future studies could explore the broader applicability of these findings by investigating whether miR-27b-3p plays a similar role in other age-related degenerative diseases. Additionally, clinical trials targeting miR-27b-3p or its downstream pathways in patients with IVDD could provide critical insights into the therapeutic potential of this approach.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant nos. 82272551 and 81772408).

Availability of data and materials

The data generated in the present study may be found in the Gene Expression Omnibus under accession number GSE205535 or at the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE205535>.

Authors' contributions

QWL designed the study, performed the Mendelian randomization analysis, and interpreted the data. CHZ conducted the experimental work, including *in vivo* and *in vitro* analyses, and contributed to data interpretation. PLJ made substantial contributions to the acquisition of data, assisted with data analysis and interpretation, and revised the manuscript critically for important intellectual content. CLS contributed to the conception and design of the study, supervised the research, provided critical revisions, and finalized the manuscript. All authors read and approved the final version of the manuscript, agreed to be accountable for all aspects of the work, and confirm their contributions are consistent with the ICMJE criteria. QWL and CHZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The animal experimental protocols were approved (approval no. IHM-AP-2024-015) by the Laboratory Animal Care and Use Committee of the Institute of Health and Medicine of Hefei Comprehensive National Science Center (Hefei, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Saberi M, Zhang X and Mobasheri A: Targeting mitochondrial dysfunction with small molecules in intervertebral disc aging and degeneration. *Geroscience* 43: 517-537, 2021.
2. Wang F, Cai F, Shi R, Wang XH and Wu XT: Aging and age related stresses: A senescence mechanism of intervertebral disc degeneration. *Osteoarthritis Cartilage* 24: 398-408, 2016.
3. Wang Y, Cheng H, Wang T, Zhang K, Zhang Y and Kang X: Oxidative stress in intervertebral disc degeneration: Molecular mechanisms, pathogenesis and treatment. *Cell Prolif* 56: e13448, 2023.
4. Xiang Q, Zhao Y and Li W: Identification and validation of ferroptosis-related gene signature in intervertebral disc degeneration. *Front Endocrinol (Lausanne)* 14: 1089796, 2023.
5. Yi J, Zhou Q, Huang J, Niu S, Ji G and Zheng T: Lipid metabolism disorder promotes the development of intervertebral disc degeneration. *Biomed Pharmacother* 166: 115401, 2023.
6. Koroth J, Buko EO, Abbott R, Johnson CP, Ogle BM, Stone LS, Ellingson AM and Bradley EW: Macrophages and intervertebral disc degeneration. *Int J Mol Sci* 24: 1367, 2023.
7. Samanta A, Lufkin T and Kraus P: Intervertebral disc degeneration: Current therapeutic options and challenges. *Front Public Health* 11: 1156749, 2023.
8. Silwal P, Nguyen-Thai AM, Mohammad HA, Wang Y, Robbins PD, Lee JY and Vo NV: Cellular senescence in intervertebral disc aging and degeneration: Molecular mechanisms and potential therapeutic opportunities. *Biomolecules* 13: 686, 2023.
9. Wang HS, Lin S and Yu HM: Exosome-mediated repair of intervertebral disc degeneration: The potential role of miRNAs. *Curr Stem Cell Res Ther* 19: 798-808, 2024.
10. Sharma Y, Saini AK, Kashyap S, Chandan G, Kaur N, Gupta VK, Thakur VK, Saini V and Saini RV: Host miRNA and immune cell interactions: Relevance in nano-therapeutics for human health. *Immunol Res* 70: 1-18, 2022.
11. Schell SL and Rahman ZSM: miRNA-mediated control of B cell responses in immunity and SLE. *Front Immunol* 12: 683710, 2021.
12. Bronevetsky Y and Ansel KM: Regulation of miRNA biogenesis and turnover in the immune system. *Immunol Rev* 253: 304-316, 2013.
13. Wythe SE, Nicolaidou V and Horwood NJ: Cells of the immune system orchestrate changes in bone cell function. *Calcif Tissue Int* 94: 98-111, 2014.
14. Sun K, Jiang J, Wang Y, Sun X, Zhu J, Xu X, Sun J and Shi J: The role of nerve fibers and their neurotransmitters in regulating intervertebral disc degeneration. *Ageing Res Rev* 81: 101733, 2022.
15. Xu H, Li J, Fei Q and Jiang L: Contribution of immune cells to intervertebral disc degeneration and the potential of immunotherapy. *Connect Tissue Res* 64: 413-427, 2023.
16. Song C, Zhou Y, Cheng K, Liu F, Cai W, Zhou D, Chen R, Shi H, Fu Z, Chen J and Liu Z: Cellular senescence-molecular mechanisms of intervertebral disc degeneration from an immune perspective. *Biomed Pharmacother* 162: 114711, 2023.
17. Risbud MV and Shapiro IM: Role of cytokines in intervertebral disc degeneration: Pain and disc content. *Nat Rev Rheumatol* 10: 44-56, 2014.
18. Sun Z, Liu ZH, Chen YF, Zhang YZ, Wan ZY, Zhang WL, Che L, Liu X, Wang HQ and Luo ZJ: Molecular immunotherapy might shed a light on the treatment strategies for disc degeneration and herniation. *Med Hypotheses* 81: 477-480, 2013.
19. Tang T, He Z, Zhu Z, Wang F, Chen H, Zhang F, Zhou J, Wang J, Li B, Liu X, *et al.*: Identification of novel gene signatures and immune cell infiltration in intervertebral disc degeneration using bioinformatics analysis. *Front Mol Biosci* 10: 1169718, 2023.
20. Zhang Y, Zhang J, Sun Z, Wang H, Ning R, Xu L, Zhao Y, Yang K, Xi X and Tian J: MAPK8 and CAPN1 as potential biomarkers of intervertebral disc degeneration overlapping immune infiltration, autophagy, and ceRNA. *Front Immunol* 14: 1188774, 2023.
21. Chen LG, Tubbs JD, Liu Z, Thach TQ and Sham PC: Mendelian randomization: Causal inference leveraging genetic data. *Psychol Med* 54: 1461-1474, 2024.

22. Fang A, Zhao Y, Yang P, Zhang X and Giovannucci EL: Vitamin D and human health: Evidence from Mendelian randomization studies. *Eur J Epidemiol* 39: 467-490, 2024.
23. Swanson SA, Labrecque J and Hernán MA: Causal null hypotheses of sustained treatment strategies: What can be tested with an instrumental variable? *Eur J Epidemiol* 33: 723-728, 2018.
24. Evans DM and Davey Smith G: Mendelian randomization: New applications in the coming age of hypothesis-free causality. *Annu Rev Genomics Hum Genet* 16: 327-350, 2015.
25. Wang P, Li Z and Ye D: Single-cell RNA-seq analysis reveals the Wnt/Ca²⁺ signaling pathway with inflammation, apoptosis in nucleus pulposus degeneration. *BMC Musculoskelet Disord* 25: 321, 2024.
26. Huan T, Rong J, Liu C, Zhang X, Tanriverdi K, Joehanes R, Chen BH, Murabito JM, Yao C, Courchesne P, *et al*: Genome-wide identification of microRNA expression quantitative trait loci. *Nat Commun* 6: 6601, 2015.
27. Orrù V, Steri M, Sidore C, Marongiu M, Serra V, Olla S, Sole G, Lai S, Dei M, Mulas A, *et al*: Complex genetic signatures in immune cells underlie autoimmunity and inform therapy. *Nat Genet* 52: 1036-1045, 2020.
28. Burgess S and Thompson SG: CRP CHD Genetics Collaboration: Avoiding bias from weak instruments in Mendelian randomization studies. *Int J Epidemiol* 40: 755-764, 2011.
29. Bowden J, Del Greco M F, Minelli C, Zhao Q, Lawlor DA, Sheehan NA, Thompson J and Davey Smith G: Improving the accuracy of two-sample summary-data Mendelian randomization: Moving beyond the NOME assumption. *Int J Epidemiol* 48: 728-742, 2019.
30. Glickman ME, Rao SR and Schultz MR: False discovery rate control is a recommended alternative to Bonferroni-type adjustments in health studies. *J Clin Epidemiol* 67: 850-857, 2014.
31. Dudbridge F: Polygenic Mendelian randomization. *Cold Spring Harb Perspect Med* 11: a039586, 2021.
32. Greco M FD, Minelli C, Sheehan NA and Thompson JR: Detecting pleiotropy in Mendelian randomisation studies with summary data and a continuous outcome. *Stat Med* 34: 2926-2940, 2015.
33. Fan C, Wang W, Yu Z, Wang J, Xu W, Ji Z, He W, Hua D, Wang W, Yao L, *et al*: M1 macrophage-derived exosomes promote intervertebral disc degeneration by enhancing nucleus pulposus cell senescence through LCN2/NF-κB signaling axis. *J Nanobiotechnology* 22: 301, 2024.
34. Lin Z, Xu G, Lu X, Liu S, Zou F, Ma X, Jiang J, Wang H and Song J: Chondrocyte-targeted exosome-mediated delivery of Nrf2 alleviates cartilaginous endplate degeneration by modulating mitochondrial fission. *J Nanobiotechnology* 22: 281, 2024.
35. Dai J, Liu J, Shen Y, Zhang B, Li C and Liu Z: Regulation of endoplasmic reticulum stress on autophagy and apoptosis of nucleus pulposus cells in intervertebral disc degeneration and its related mechanisms. *PeerJ* 12: e17212, 2024.
36. Gao W, Bao J, Zhang Y, He D, Zhang L, Zhang J, Pan H and Wang D: Injectable kaempferol-loaded fibrin glue regulates the metabolic balance and inhibits inflammation in intervertebral disc degeneration. *Sci Rep* 13: 20001, 2023.
37. Chen X, Zhang A, Zhao K, Gao H, Shi P, Chen Y, Cheng Z, Zhou W and Zhang Y: The role of oxidative stress in intervertebral disc degeneration: Mechanisms and therapeutic implications. *Ageing Res Rev* 98: 102323, 2024.
38. Guo W, Mu K, Geng JC, Xing HY, Dong Y, Liu WD, Wang SC, Shi JX, Xing BR, Zhao JY and Li XM: ATF1 and miR-27b-3p drive intervertebral disc degeneration through the PPARγ/NF-κB signaling axis. *Commun Biol* 8: 751, 2025.
39. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
40. Rouas R, Merimi M, Najar M, El Zein N, Fayyad-Kazan M, Berehab M, Agha D, Bron D, Burny A, Rachidi W, *et al*: Human CD8⁺ CD25⁺ CD127^{low} regulatory T cells: microRNA signature and impact on TGF-β and IL-10 expression. *J Cell Physiol* 234: 17459-17472, 2019.
41. Diener C, Keller A and Meese E: Emerging concepts of miRNA therapeutics: From cells to clinic. *Trends Genet* 38: 613-626, 2022.
42. Ferragut Cardoso AP, Banerjee M, Nail AN, Lykoudi A and States JC: miRNA dysregulation is an emerging modulator of genomic instability. *Semin Cancer Biol* 76: 120-131, 2021.
43. Vafadar A, Shirazi-Tehrani E, Vosough P, Khalili Alashti S, Kargar Jahromi H, Bagheri Lankarani K, Savardashtaki A and Ehtiati S: Non-coding RNAs in celiac disease. *Clin Chim Acta* 576: 120417, 2025.
44. Wang Y, Kang J, Guo X, Zhu D, Liu M, Yang L, Zhang G and Kang X: Intervertebral disc degeneration models for pathophysiology and regenerative therapy-benefits and limitations. *J Invest Surg* 35: 935-952, 2022.
45. Hu A, Xing R, Jiang L, Li Z, Liu P, Wang H, Li X and Dong J: Thermosensitive hydrogels loaded with human-induced pluripotent stem cells overexpressing growth differentiation factor-5 ameliorate intervertebral disc degeneration in rats. *J Biomed Mater Res B Appl Biomater* 108: 2005-2016, 2020.
46. Kilkenny C, Browne WJ, Cuthill IC, Emerson M and Altman DG: Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *PLoS Biol* 8: e1000412, 2010.
47. Griffith JF, Wang YXJ, Antonio GE, Choi KC, Yu A, Ahuja AT and Leung PC: Modified Pfirrmann grading system for lumbar intervertebral disc degeneration. *Spine (Phila Pa 1976)* 32: E708-E712, 2007.
48. Genedy HH, Humbert P, Laoulaou B, Le Moal B, Fusellier M, Passirani C, Le Visage C, Guicheux J, Lepeltier É and Clouet J: MicroRNA-targeting nanomedicines for the treatment of intervertebral disc degeneration. *Adv Drug Deliv Rev* 207: 115214, 2024.
49. Wang Y, Deng M, Wu Y, Zheng C, Zhang F, Guo C, Zhang B, Hu C, Kong Q and Wang Y: A multifunctional mitochondria-protective gene delivery platform promote intervertebral disc regeneration. *Biomaterials* 317: 123067, 2025.
50. Xu YQ, Zhang ZH, Zheng YF and Feng SQ: Dysregulated miR-133a mediates loss of type II collagen by directly targeting matrix metalloproteinase 9 (MMP9) in human intervertebral disc degeneration. *Spine (Phila Pa 1976)* 41: E717-E724, 2016.
51. Li X, Xu M, Ding L and Tang J: MiR-27a: A novel biomarker and potential therapeutic target in tumors. *J Cancer* 10: 2836-2848, 2019.
52. Zhang J, Cao Z, Yang G, You L, Zhang T and Zhao Y: MicroRNA-27a (miR-27a) in solid tumors: A review based on mechanisms and clinical observations. *Front Oncol* 9: 893, 2019.
53. Su L, Li R, Zhang Z, Liu J, Du J and Wei H: Identification of altered exosomal microRNAs and mRNAs in Alzheimer's disease. *Ageing Res Rev* 73: 101497, 2022.
54. Speiser DE, Chijioke O, Schaeuble K and Münz C: CD4⁺ T cells in cancer. *Nat Cancer* 4: 317-329, 2023.
55. Sun L, Su Y, Jiao A, Wang X and Zhang B: T cells in health and disease. *Signal Transduct Target Ther* 8: 235, 2023.
56. Zhu J, Yamane H and Paul WE: Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 28: 445-489, 2010.
57. Borst J, Ahrends T, Bąbala N, Melief CJM and Kastenmüller W: CD4⁺ T cell help in cancer immunology and immunotherapy. *Nat Rev Immunol* 18: 635-647, 2018.
58. Thomas R, Qiao S and Yang X: Th17/Treg imbalance: Implications in lung inflammatory diseases. *Int J Mol Sci* 24: 4865, 2023.
59. Sakaguchi S, Mikami N, Wing JB, Tanaka A, Ichiyama K and Ohkura N: Regulatory T cells and human disease. *Annu Rev Immunol* 38: 541-566, 2020.
60. Churov AV, Mamashov KY and Novitskaia AV: Homeostasis and the functional roles of CD4⁺ Treg cells in aging. *Immunol Lett* 226: 83-89, 2020.
61. Wei B, Zhao Y, Li W, Zhang S, Yan M, Hu Z and Gao B: Innovative immune mechanisms and antioxidative therapies of intervertebral disc degeneration. *Front Bioeng Biotechnol* 10: 1023877, 2022.
62. Dou Y, Zhang Y, Liu Y, Sun X, Liu X, Li B and Yang Q: Role of macrophage in intervertebral disc degeneration. *Bone Res* 13: 15, 2025.
63. Lee S and Lee W: A review of Mendelian randomization: Assumptions, methods, and application to obesity-related diseases. *J Obes Metab Syndr* 34: 14-26, 2025.
64. Chen B, Yan Y, Wang H and Xu J: Association between genetically determined telomere length and health-related outcomes: A systematic review and meta-analysis of Mendelian randomization studies. *Ageing Cell* 22: e13874, 2023.
65. Ni F, Liu X and Wang S: Impact of negative emotions and insomnia on sepsis: A mediation Mendelian randomization study. *Comput Biol Med* 180: 108858, 2024.

