



Article

Single-Nucleus RNA Sequencing Reveals SPP1⁺ Macrophages Induce Cardiomyocyte Apoptosis to Promote Atrial Fibrillation Susceptibility

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Abstract

Atrial fibrillation (AF) is closely linked to atrial remodeling, while its underlying immune mechanisms remain elusive. This study sought to investigate the role of SPP1⁺ macrophages in the development and progression of AF and further elucidate the underlying mechanisms. Single-nucleus RNA sequencing was performed on right atrial tissues from 3 patients with persistent AF and 3 with sinus rhythm (all with rheumatic valvular heart disease). The results revealed significant immune cell infiltration in AF atrial tissues, with a marked increase in the proportion of SPP1⁺ macrophages, which exhibited the strongest intercellular communication with cardiomyocytes. Phenotypic scoring indicated that apoptosis was the dominant mode of cardiomyocyte death in AF. Immunohistochemical and Western blot analyses confirmed elevated levels of pro-apoptotic proteins (Bax, Cleaved-Caspase3) and reduced levels of the anti-apoptotic protein Bcl2 in AF tissues. In a mouse model with macrophage-specific SPP1 overexpression, increased AF inducibility and duration were observed, accompanied by enhanced cardiomyocyte apoptosis. In vitro co-culture experiments using SPP1-overexpressing RAW264.7 macrophages and HL-1 cardiomyocytes confirmed that SPP1⁺ macrophages could induce cardiomyocyte apoptosis. Mechanistically, KEGG and GSEA analyses identified downregulation of the PI3K/AKT pathway in AF. Treatment with the PI3K/AKT activator Recilisib reversed apoptosis and restored p-PI3K/p-AKT levels in HL-1 cells co-cultured with SPP1-overexpressing RAW264.7 macrophages. These findings demonstrate that SPP1⁺ macrophages accumulate in atrial tissues of AF patients and induce cardiomyocyte apoptosis by downregulating the PI3K/AKT pathway, thereby increasing AF susceptibility.

Keywords: atrial fibrillation; SPP1⁺ macrophages; cardiomyocyte apoptosis; PI3K/AKT signaling pathway



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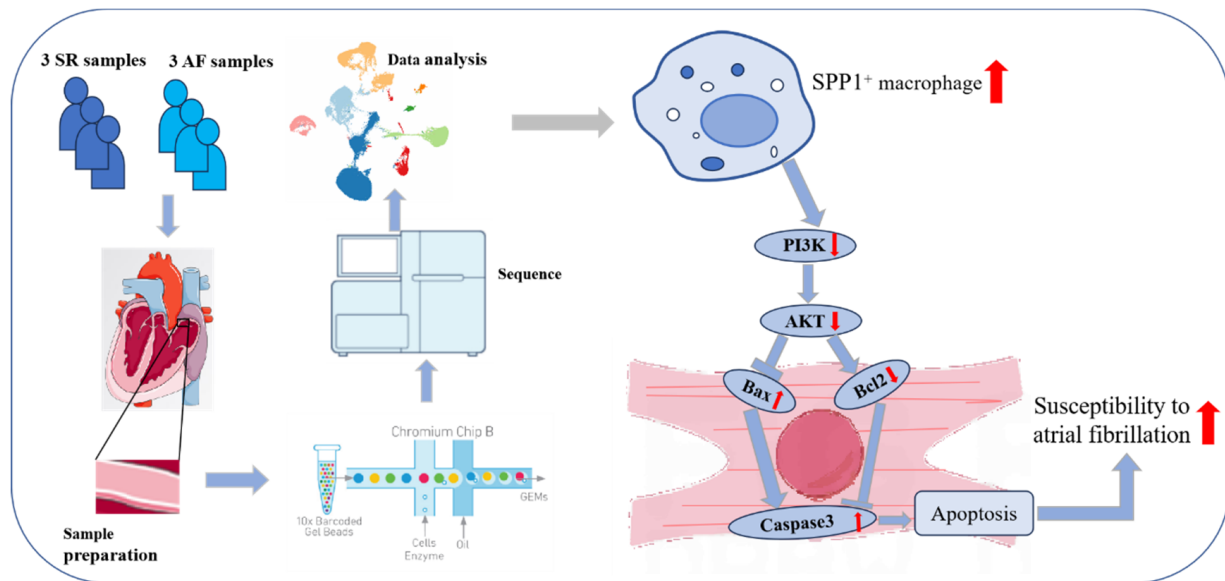
1. Background

Atrial fibrillation (AF) is the most common type of arrhythmia, characterized by an electrical disorder in the atrium. The irregular fibrillation, contraction, and relaxation of the atrium lead to dysfunction in atrial pumping, significantly increasing the risk of adverse events such as stroke, heart failure, cognitive impairment, and death in patients with AF [1]. Atrial fibrillation (AF) represents the most prevalent sustained cardiac arrhythmia worldwide, with its epidemiological burden escalating globally [2]. The estimated global prevalence was 50 million in 2020 [2,3]. Currently, the treatment strategies for AF mainly

include heart rate control, rhythm control (e.g., pharmacological cardioversion, catheter ablation), and anticoagulant therapy [4]. However, the efficacy of existing treatments remains unsatisfactory for persistent or long-standing AF, with a high recurrence rate. The fundamental reason for this clinical dilemma is our insufficient understanding of the underlying molecular mechanisms that govern the occurrence and maintenance of AF. Therefore, in-depth investigation into the novel mechanisms underlying AF and identification of novel intervention targets are of great scientific significance and clinical value.

The traditional perspective maintains that the pathological foundation of AF is rooted in both atrial electrical remodeling and structural remodeling [5–7]. With the continuous deepening of research, more and more evidence indicates that the inflammatory process may be the core of the pathogenesis of AF, and immune cells have played a significant role in its onset and progression [8]. Previous studies have demonstrated a significant increase in the prevalence of inflammatory macrophages within the atrial tissue of patients diagnosed with atrial fibrillation [9]. As pivotal sentinels of the innate immune system, macrophages exhibit pronounced heterogeneity and functional plasticity within the cardiac microenvironment [10]. Recent studies have evolved to identify that the crosstalk between macrophages and cardiomyocytes significantly contributes to cardiac remodeling [11–13]. Studies have found that a substantial rise in the abundance of mononuclear phagocyte and dendritic cell (MP/DC) clusters within the atrial tissue of patients with AF, concomitant with a relative reduction in the proportions of endothelial and mural cells [14]. Bioinformatics analysis suggests that inflammatory cells such as macrophages, mast cells, and neutrophils significantly infiltrate the atrial tissue of AF patients [15]. Similarly, in an immunohistochemical analysis of surgically obtained right atrial appendage specimens, Smorodinova et al. reported a higher density of inflammatory infiltrates in AF patients compared to those with sinus rhythm (SR). This infiltrate was primarily composed of cells from the mononuclear phagocyte system, notably macrophages [16]. The recent emergence of high-resolution technologies, particularly single-nucleus RNA sequencing (snRNA-seq), has enabled the identification of a distinct macrophage subset characterized by high expression of secreted phosphoprotein 1 (SPP1; osteopontin) across various models of heart disease [17]. This macrophage subset is characterized by potent pro-inflammatory and pro-fibrotic activities. Through the secretion of key effector molecules, including SPP1 and TGF- β , this subset engages with cardiac fibroblasts, promoting excessive collagen deposition and interstitial fibrosis. This process ultimately establishes a structural substrate conducive to reentrant excitation circuits [18]. The stability of the atrial architecture is contingent upon not only the integrity of the extracellular matrix (ECM) but also, critically, on the preservation of cardiomyocyte viability. Significant cardiomyocyte apoptosis results in atrial wall thinning, creates a substrate for heterogeneous electrical conduction, and can directly elicit ectopic excitation; these combined effects thereby initiate and perpetuate AF [19].

In this study, we first performed snRNA-seq analysis on atrial tissues from patients with AF and sinus rhythm (SR) to characterize changes in cell subsets. Our data revealed a significant accumulation of SPP1⁺ macrophages in AF atrial tissues, with functional annotations indicating their involvement in pro-inflammatory responses, extracellular matrix regulation, and extracellular secretion. Building on these findings, we aimed to elucidate the key mechanisms by which SPP1⁺ macrophages interact with cardiomyocytes to induce cardiomyocyte apoptosis, using a combination of clinical samples, animal models, and cell co-culture systems (Scheme 1). It is expected to provide a new perspective for understanding the immunometabolic mechanism of atrial fibrillation (AF) and offer an important theoretical basis as well as potential therapeutic targets for delaying AF progression through targeted intervention of SPP1 or related pathways.



Scheme 1. Single-nucleus RNA Sequencing Reveals SPP1⁺ Macrophages Induce Cardiomyocyte Apoptosis to Promote Atrial Fibrillation Susceptibility.

2. Materials and Methods

2.1. Human Subjects

The study protocol received approval from the Ethics Committee of the Second Affiliated Hospital of Nanchang University. All procedures were performed in accordance with the ethical standards of the Declaration of Helsinki. Tissue samples were obtained from the right atrial tissue of patients with rheumatic valvular heart disease who underwent mitral valve replacement surgery in the Department of Cardiothoracic and Vascular Surgery, the Second Affiliated Hospital of Nanchang University. The cohort consisted of 5 patients with persistent atrial fibrillation (AF) and 5 patients in sinus rhythm (SR). A portion of the freshly resected tissue was fixed in 4% paraformaldehyde (P0099, Beyotime, Shanghai, China), while the remainder was immediately snap-frozen in liquid nitrogen for further processing. Three samples per group were randomly selected for single-nucleus RNA sequencing, and all 5 samples per group were used for subsequent experimental validation to confirm the sequencing findings.

2.2. Animals

All animal experiments were approved and conducted in full compliance with international ethical standards, including the UK Animals (Scientific Procedures) Act 1986, the EU Directive 2010/63/EU, and the US National Research Council's Guide for the Care and Use of Laboratory Animals. Male C57BL/6J mice (7 weeks old, weighing 21.5–22.5 g) were obtained from Huachuang Sinuo (Taizhou, China). All animals were housed in a specific pathogen-free (SPF) facility with strictly controlled temperature (22 ± 2 °C), humidity (45–65%), and a 12 h light-dark cycle, using individually ventilated cages (IVC) equipped with disinfected bedding. After 1 week of acclimatization, mice received an intravenous injection of an adeno-associated virus 9 (AAV9) vector to induce macrophage-specific SPP1 overexpression. Thirty male C57BL/6J mice were randomly allocated into three groups: the untreated group (WT, $n = 10$), the viral AAV9-injected group (AAV9-SPP1, $n = 10$), and the negative control-injected group (Vector, $n = 10$). A single tail vein injection of AAV9 (HBAAV2/9-F4/80-m-Spp1-3xflag-mcherry) or negative control (HBAAV2/9-F4/80-mcherry) (Hanbio Biotechnology, Shanghai, China) was administered at a dose of 5×10^{10} viral genomes per mouse. Three weeks post-transfection, the suc-

successful construction of the overexpression animal model was verified. Subsequently, atrial fibrillation induction experiments were conducted at four weeks post-transfection.

Mice were anesthetized via inhalation of isoflurane (3% for induction, 1–1.5% for maintenance), and a 27-gauge needle was inserted subcutaneously into the forelimb to acquire surface electrocardiograms (ECG). An octapolar electrophysiology catheter (1.1 F; Transonic Scisense, London, ON, Canada) was introduced into the right jugular vein and fluoroscopically guided to the right atrium. It was then advanced to position the distal electrodes within the right ventricular apex, ensuring stable contact for programmed electrical stimulation. A standardized rapid atrial pacing protocol was employed to assess atrial fibrillation (AF) inducibility. Stimuli were delivered through the intracardiac catheter electrodes using an automated stimulator (MADLAB-4C/501H data acquisition system; Zhongshi Dichuang Technology Co., Ltd., Beijing, China). The protocol comprised 11 consecutive burst pacing trains, each lasting 6 s. The first train had a cycle length (CL) of 40 ms, with the CL decreasing by 2 ms in each subsequent train, down to a minimum of 20 ms, in accordance with established methods. After allowing a 5 min stabilization period following each full series, the entire pacing protocol was repeated twice for a total of three iterations. Successful induction was defined as a rapid, irregular atrial rhythm lasting more than 1 s, observed via direct atrial activation recording during the subsequent 30 min recording period. Each animal underwent only one blinded experiment. All experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Nanchang University (approval No. NCULAE-20250901005).

2.3. Cell Culture

RAW264.7 (TCM13) and HL-1 (GNM46) cells were both purchased from the Cell Bank of the Chinese Academy of Sciences and cultured in DMEM medium containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific). All cells were incubated in a humidified environment at 37 °C with 5% carbon dioxide. To establish SPP1-overexpressing macrophages, RAW264.7 cells were cultured in 6-well plates. Upon reaching approximately 70% confluence, cells were transduced with lentiviral vectors purchased from HanBio (Shanghai, China). The experimental group received lentiviruses carrying the SPP1 gene, while the control group was transduced with non-targeting control vectors. Transduction was performed in medium supplemented with 5 µg/mL polybrene at a multiplicity of infection (MOI) of 100 for 24 h, after which the virus-containing medium was replaced with fresh complete medium. Macrophages and HL-1 cardiomyocytes were co-cultured in a transwell system for 24 h, with macrophages in the upper chamber and HL-1 cells in the lower chamber. Subsequently, the HL-1 cells were collected for analysis.

2.4. Single-Nucleus RNA Sequencing and Data Analysis

2.4.1. Preparation of Single-Nucleus Suspension

For single-nucleus RNA sequencing (snRNA-seq), frozen atrial tissue samples were processed. Frozen tissue was dounced in 3 mL of lysis buffer (10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂ and 0.05% (v/v) NP-40 detergent), 10 times with a loose pestle and an additional 5 times with the tight pestle (glass dounce homogenizer). The sample was left to lyse in a total of 5 mL of buffer for 5 min, after which 5 mL of wash buffer (10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 1% bovine serum albumin (BSA), 1 mM DTT, RNase inhibitor 1 U/µL, Nuclease-free Water) was added. The sample was passed through a 30-µm cell strainer and spun for 5 min at 500× g. After pelleting, the nuclei were resuspended in 10 mL of wash buffer by pipetting up and down 10 times. After 3 washes, the nuclei were resuspended in 1 mL of wash buffer and mixed with 25% OptiPrep (D1556,

Sigma-Aldrich, St. Louis, MO, USA) and layered on a 29% OptiPrep cushion and spun for 30 min at $10,000\times g$. Nuclei were resuspended in wash buffer and 3 washes. A 10 μL aliquot of the nuclear suspension was taken for analysis using a fluorescence-based cell counter with AO/PI staining to determine the concentration, viability, background quality, and size distribution of the single-nuclei suspension. Nuclear morphology was further examined under a microscope. Finally, the nuclei were resuspended in Nuclei Resuspension Buffer (Nuclei buffer (20x stock, 10X Genomics, Pleasanton, CA, USA) 1x, 1 mM DTT, RNase inhibitor 1 U/ μL , Nuclease-free Water) at a concentration of approximately $1 \times 10^6/\text{mL}$.

2.4.2. Construction and Sequencing of Single-Nucleus Libraries

The quality, concentration, and viability (>80%) of the single-nucleus suspension were assessed using an automated cell counter from Bio-Rad (Hercules, CA, USA). The prepared cell suspension (800–1200 cells/ μL) was mixed with reagent cocktail, single-nucleus 5' GEM beads (with unique barcodes), and oil droplets, then loaded into the microfluidic channel of the 10X Genomics Chromium system to form oil-encapsulated single-nucleus microreactors (GEMs). Within the GEMs, cells were lysed to release mRNA, which was immediately captured by barcoded oligonucleotides on the beads and reverse-transcribed into cDNA. The quality of the library was evaluated using Qubit quantification reagents (Thermo Fisher Scientific) to ensure it met library preparation standards. Library quality control was performed based on electrophoretic analysis of DNA content and fragment size distribution, with the following criteria applied: (1) Total DNA amount > 100 ng as measured by Qubit assay. (2) A dominant peak within the 300 bp to 600 bp range. (3) Proportion of fragments between 600 bp and 5000 bp < 10%. Libraries were sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) using a 150 bp paired-end strategy.

2.4.3. Data Processing

The downloaded fastq files were quantitatively analyzed using Cellranger 3.0.2 to convert fastq data into a gene-barcode matrix. The specific steps were as follows: first, barcode, UMI, and RNA read data were read. Read lengths were trimmed to retain only the required length. Read 1 was trimmed to 28 bp and Read 2 was trimmed to 98 bp prior to alignment to the reference genome. The STAR algorithm was used to align RNA reads, and gene and transcript labels for RNA reads were annotated. Next, UMI sequences were corrected, UMI counts were calculated, cell-associated barcodes were selected, and finally, a single-nucleus expression matrix was generated. The single-nucleus expression matrix was read, and low-quality data were filtered out using UMI counts (5000–25,000), number of genes (>200), and mitochondrial gene proportion (<20%) to retain high-quality data. The NormalizeData function of the Seurat package (version 4.3.0) was used to normalize the data, correcting for differences in sequencing library sizes among different cells. The FindVariableFeatures function was used to identify the 2000 most significant highly variable genes in each sample for subsequent analyses. The ScaleData function was applied to add z-scores to the data processed by NormalizeData, scaling the expression levels of each gene to the same range to enable comparability of expression levels for the same gene. The RunPCA function was used for dimensionality reduction, converting high-dimensional data into fewer dimensions while retaining the most important variation information for subsequent visualization and analysis. Meanwhile, integration of multi-sample data may introduce non-biological differences. The RunHarmony function was used to adjust the similarity matrix among samples, reducing batch effects so that cells from different samples could be better integrated in low-dimensional space. Subsequently, the RunUMAP and FindClusters functions were used for dimensionality reduction and

clustering. Uniform Manifold Approximation and Projection (UMAP) plots were used to visualize single-nucleus cluster clustering.

2.4.4. Cell-Immune Cells Subsets

The CellChat R package (v1.6.1) was used to analyze the interaction network between cardiomyocytes and immune cell subsets. Ligand-receptor genes highly expressed in each cell population were screened. Ligand-receptor pairs were defined, and their expression data were contextualized within a protein–protein interaction (PPI) network; this integration facilitated the identification of statistically significant cell–cell communication events. Interactions involving upregulated ligands or receptors were prioritized in the analysis.

2.4.5. Gene Set Scoring

Gene set scoring was performed to calculate an enrichment or activity score for a given set of target genes. In this study, the AddModuleScore function in the Seurat package was used to assign scores to gene sets of interest, followed by inter-group comparisons. The AddModuleScore algorithm works as follows: the mean expression of all genes is first calculated, and genes are then binned based on these mean expression values. The distribution of target genes across bins is examined, and a corresponding set of background genes is randomly selected from the same bins. For each cell, the average expression of the target genes is computed and then subtracted from the average expression of the background genes to yield the final module score. Marker genes can be found in Document S1.

2.5. Western Blot

Total protein was extracted from harvested cells or snap-frozen heart tissue samples using the same RIPA lysis buffer supplemented with a mixture of 1% protease inhibitors and 1% phosphatase inhibitors, and protein concentration was determined using a BCA kit (Beyotime). Protein samples (approximately 20–30 µg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked at room temperature for 1 h in Tris-buffered saline containing 0.1% Tween-20 (TBST). For the detection of non-phosphorylated proteins, blocking was performed with TBST containing 5% (*w/v*) non-fat milk. For phosphorylated proteins, membranes were blocked with TBST containing 5% (*w/v*) bovine serum albumin (BSA). They were then incubated overnight at 4 °C with primary antibodies, including rabbit anti-Bax (50599-2-Ig, 1:20000, Proteintech, Wuhan, China), rabbit anti-Bcl2 (AF6139, 1:1000, Affinity, Liyang, China), rabbit anti-Cleaved-caspase 3 (25128-1-AP, 1:1000, Proteintech), rabbit anti-SPP1 (22952-1-AP, 1:2000, Proteintech), mouse anti-PI3K (60225-1-Ig, 1:5000, Proteintech), rabbit anti-pPI3K(17366,1:1000, CellSignalingTechnology, Danvers, MA, USA), rabbit anti-AKT (10176-2-AP, 1:2000, Proteintech), rabbit anti-pAKT (80455-1-RR, 1:2000, Proteintech) and mouse anti-GAPDH (60004-1-Ig, 1:10000, Proteintech). Following incubation with primary antibodies and subsequent washes, membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Finally, immunoreactive protein bands were visualized with the BIO-RAD ChemiDoc™ XRS+ imaging system, and band intensities were quantified using Image J software(v 2.3.0).

2.6. qPCR Assays

Total RNA was extracted from heart tissues or cells using the TRIzol reagent kit (15596026CN, Thermo Fisher). Quantitative polymerase chain reaction (qPCR) was performed on an Applied Biosystems ViiA 7 instrument (Thermo Fisher Scientific, Waltham, MA, USA) using the SYBR Green Premix Pro Taq HS qPCR Kit (Takara Bio, Kusatsu, Japan). The relative expression levels with GAPDH as the reference were calculated from three

biological replicates using the $2^{-\Delta\Delta CT}$ method. All qPCR primers were designed and synthesized by RIBOBIO (Guangzhou, China). Prior to data analysis, the expression stability of the selected reference gene GAPDH was verified by comparing its cycle threshold (Ct) values across all experimental conditions. No statistically significant difference in GAPDH expression was detected among the experimental groups, confirming its suitability as a normalization control in this study. No statistically significant variation in GAPDH expression was detected between experimental groups ($p > 0.05$) (Figure S1), confirming its suitability for normalization in this study. The sequences are listed below:

Mus-GAPDH F 5'-GAACGGGAAGCTCACTGG-3'
 Mus-GAPDH R 5'-GCCTGCTTCACCACCTTCT-3'
 Mus-SPP1 F 5'-CCATGAGTCAAGTCAGCTGGATG-3'
 Mus-SPP1 R 5'-CTTGTGGCTCTGATGTTCCAGG-3'
 Homo-GAPDH F 5'-CGAGGTGATAGTGTGGTTTATGG-3'
 Homo-GAPDH R 5'-GCACCATTCAACTCCTCGCTTTC-3'
 Homo-SPP1 F 5'-CGAGGTGATAGTGTGGTTTATGG-3'
 Homo-SPP1 R 5'-GCACCATTCAACTCCTCGCTTTC-3'

2.7. Histological Analysis

Human right atrial tissues were obtained from the Second Affiliated Hospital of Nanchang University. Tissues were fixed, processed for paraffin embedding, and sectioned. To evaluate apoptosis, immunohistochemistry was employed to assess the immunoreactivity of the proteins Bax, Bcl2, and Cleaved-Caspase3 in tissue sections. Prior to staining, the sections were dewaxed in xylene, then rehydrated in 100%, 95%, and 70% ethanol sequentially. This was followed by antigen retrieval using a microwave method. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ in methanol for 15 min at room temperature. After washing with PBS, non-specific binding sites were blocked with 5% BSA for 1 h at room temperature. The sections were incubated with primary antibodies at 4 °C overnight, then with horseradish peroxidase (HRP)-labeled secondary antibodies at room temperature for 1 h. The immunoreactivity was visualized with DAB chromogen, and the nuclei were counterstained with hematoxylin. Finally, the sections were dehydrated, cleared, and mounted with neutral balsam for microscopic observation. For IHC sections stained with hematoxylin-DAB (H-DAB), five fields of view per sample were captured using Fiji software (v2.3.0). The DAB channel was separated by color deconvolution, and the threshold was set as the mean optical density of the negative control plus three standard deviations. The number of DAB-positive cells and the total number of cells stained with hematoxylin were counted, and the percentage was calculated as: (number of positive cells/total number of cells) $\times$ 100%. The average value from all fields of each sample was used for subsequent intergroup statistical analysis. During the calculation of the DAB-positive area ratio, the analyst was kept blinded to the sample grouping.

Atrial tissue samples from mice were collected after inducing atrial fibrillation. Immunofluorescent staining was used to detect the expression and distribution of apoptosis-related proteins (Bax, Bcl2, Cleaved-Caspase3). Mouse atrial tissue sections were permeabilized with 0.1% Triton X-100 (Solarbio, Beijing, China) in PBS for 20 min, then blocked with 2% BSA (Solarbio) containing HNEpC for 30 min at room temperature. Subsequently, the sections were incubated with primary antibodies overnight at 4 °C, and with secondary antibodies for 1 h at room temperature, respectively. Nuclei were counterstained with DAPI.

2.8. Apoptosis Detection

Cell apoptosis induced by co-culture of RAW264.7 and HL-1 cells was assessed using an Annexin V-FITC/PI double-staining kit (Yeasen, Shanghai, China). RAW264.7

(4×10^5 cells/mL) and HL-1 (4×10^5 cells/mL) were co-cultured in a transwell chamber (4 μ m) for 24 h, then HL-1 cells in the lower chamber were collected. The cells were washed with PBS, digested with trypsin, and resuspended in 0.5 mL of Annexin binding buffer. The cells were stained in binding buffer containing Annexin V-FITC and PI for 15 min, followed by analysis via flow cytometry.

2.9. Statistical Analyses

All data are presented as the mean $\pm$ standard deviation (SD). The normality of distribution was assessed using the Shapiro–Wilk test, and the homogeneity of variances was verified. For comparisons among two or more groups that met these parametric assumptions, one-way or two-way analysis of variance (ANOVA) was performed, as appropriate, followed by Tukey’s post hoc test for multiple comparisons. When the assumptions for parametric tests were not satisfied, non-parametric tests were employed. A *p*-value of less than 0.05 was considered statistically significant. All statistical analysis was conducted using Graphpad Prism 8.

3. Results

3.1. *snRNA-Seq Revealed That the Communication Between SPP1⁺ Macrophages and Cardiomyocytes Is the Strongest*

A total of 6 right atrial tissue samples were obtained from patients undergoing cardiac surgery for rheumatic valvular heart disease, which consisted of three patients with persistent AF and three control patients with SR. The mean ages of the two groups of patients who provided the samples were 52.2 and 54.4 years old, and the mean body mass indexes were 21.61 and 22.60 Kg/m², respectively, with no statistically significant differences (*p* < 0.05). The average values of white blood cell (WBC) count, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) in both groups were within the normal range, and there were no differences (*p* < 0.05) between the groups, indicating that neither group had rheumatic activity (Table S1). After quality control with Cell Ranger, the study obtained single-nucleus quality control data for 6 right atrial samples. All Q30 metrics are above 90%, which means the probability of base miscalling is less than 0.1% (Table S2).

After rigorous quality control, batch correction, dimensionality reduction, and clustering, 121,369 high-quality cells were retained for downstream analysis. Uniform Manifold Approximation and Projection (UMAP) was employed for nonlinear dimensionality reduction and visualization, revealing well-defined cellular clusters within the right atrial tissue. Cell clusters visualized in the UMAP were annotated based on the expression of canonical cell type-specific marker genes. This analysis identified eight major cell subpopulations (Figure 1A). Patients with AF exhibited significant alterations in the proportions of multiple immune cell subsets. Compared with the SR group, the proportions of myeloid cells and lymphocytes in atrial tissues of the AF group were increased (*p* < 0.05), suggesting that there was significant immune cell infiltration in the right atrial myocardial tissue of AF patients. The proportion of cardiomyocytes in the AF group was significantly reduced (*p* < 0.05), which indicates that cardiomyocyte death may occur in the right atrial myocardial tissue of AF patients (Figure 1B). Compared with the SR group, the phenotypic scores of cardiomyocyte apoptosis, pyroptosis, ferroptosis, autophagy, necroptosis, immunogenic cell death, extracellular trap formation, and endocytic cell death in the AF group were significantly increased (*p* < 0.05). Notably, subsequent analysis revealed that the phenotypic score for apoptosis significantly surpassed that of all other programmed cell death pathways examined. This finding indicates that apoptotic cell death represents the predominant programmed cell death mechanism in right atrial cardiomyocytes of AF patients (Figure 1C). Myeloid cell populations were categorized into distinct subsets based on the

expression of canonical signature genes, as follows: resident macrophages (*MRC1*, *C1QA*, *CD14*), macrophages with high *CCR2* expression (*CCR2*, *C1QA*, *CD14*), macrophages with high *SPP1* expression (*SPP1*, *C1QA*, *CD14*), monocytes (*WDFY4*, *S100A11*, *TIMP1*), and mast cells (*TPSB2*, *TPSAB1*). Lymphocytes are further divided into $CD8^+$ T cells (*CD8B*, *CD3D*, *CD3E*, *CD3G*, *CD96*), $CD4^+$ naive T cells (*CD4*, *CD3D*, *CD3E*, *CD3G*, *CD96*), natural killer (NK) cells (*KLRD1*, *NCAM1*, *FCGR3A*), natural killer T (NKT) cells (*NCAM1*, *CD3D*, *CD3E*, *CD3G*), and B cells (*IGKC*, *BLK*, *MS4A1*) (Figure 1D). As is shown in Figure 1E, compared with the SR group, the proportions of $CCR2^+$ macrophages, $SPP1^+$ macrophages, $CD4^+$ naive T cells, $CD8^+$ T cells, NK cells, and NKT cells were increased in the AF group, suggesting that immune responses mediated by both macrophages and T cells are closely associated with the pathogenesis of AF ($p < 0.05$). To evaluate the immune activity of different immune cell subsets in AF, gene sets of cytotoxic molecules, antigen-presenting molecules, and proinflammatory cytokines included in the Molecular Signatures Database (MsigDB) were incorporated for scoring and comparison. As demonstrated in Figure 1F, $SPP1^+$ macrophages from the AF group exhibited significantly higher expression levels of antigen-presenting molecules, cytotoxic molecules, and pro-inflammatory molecules compared to those from the SR group ($p < 0.05$). Analysis of ligand-receptor interactions revealed complex crosstalk between macrophages and cardiomyocytes. Unsupervised clustering analysis was performed on all cardiomyocytes (CMs) across samples. Differential gene expression analysis was conducted using the FindMarkers function in R, followed by GO biological function enrichment analysis. All CMs could be further subdivided into five distinct subpopulations (CM1–CM5), each exhibiting a unique gene expression pattern based on their differentially expressed genes. As illustrated in Figure 1G, these signaling pathways were significantly augmented in the AF group compared to the SR group. The results of the cell communication bubble plot showed that the cell communication intensity between $SPP1^+$ macrophages and cardiomyocytes was the highest (Figure 1H).

3.2. Validation of Apoptosis in Human Right Atrial Tissues Between the SR and the AF

To confirm that apoptosis is the predominant form of cardiomyocyte death in the right atrial tissue of patients with AF, immunohistochemical staining was performed. In the AF group, myocardial tissues exhibited attenuated Bcl2 expression, corresponding to weak positive immunostaining predominantly observed in cardiomyocytes and the interstitial compartment. In contrast, myocardial tissues from the SR group displayed robust Bcl2 expression, characterized by intense, widespread immunostaining ($p < 0.05$). Compared with the SR group, the expression levels of apoptosis-related proteins Bax and Cleaved-Caspase3 in myocardial tissue of the AF group were significantly increased ($p < 0.05$), with significantly enhanced staining intensity and a broader distribution range (Figure 2A–D). The results of Western blot showed that compared with the SR, the protein levels of apoptosis-related proteins Cleaved-Caspase3 and Bax in the right atrial tissue of the AF were significantly increased ($p < 0.05$), while the protein level of the anti-apoptotic protein Bcl2 was significantly decreased ($p < 0.05$) (Figure 2E–G). Western blotting and quantitative PCR analyses confirmed that the expression of *SPP1* was significantly higher in cardiac tissues from the AF group compared to the SR control group ($p < 0.05$) (Figure 2H–J).

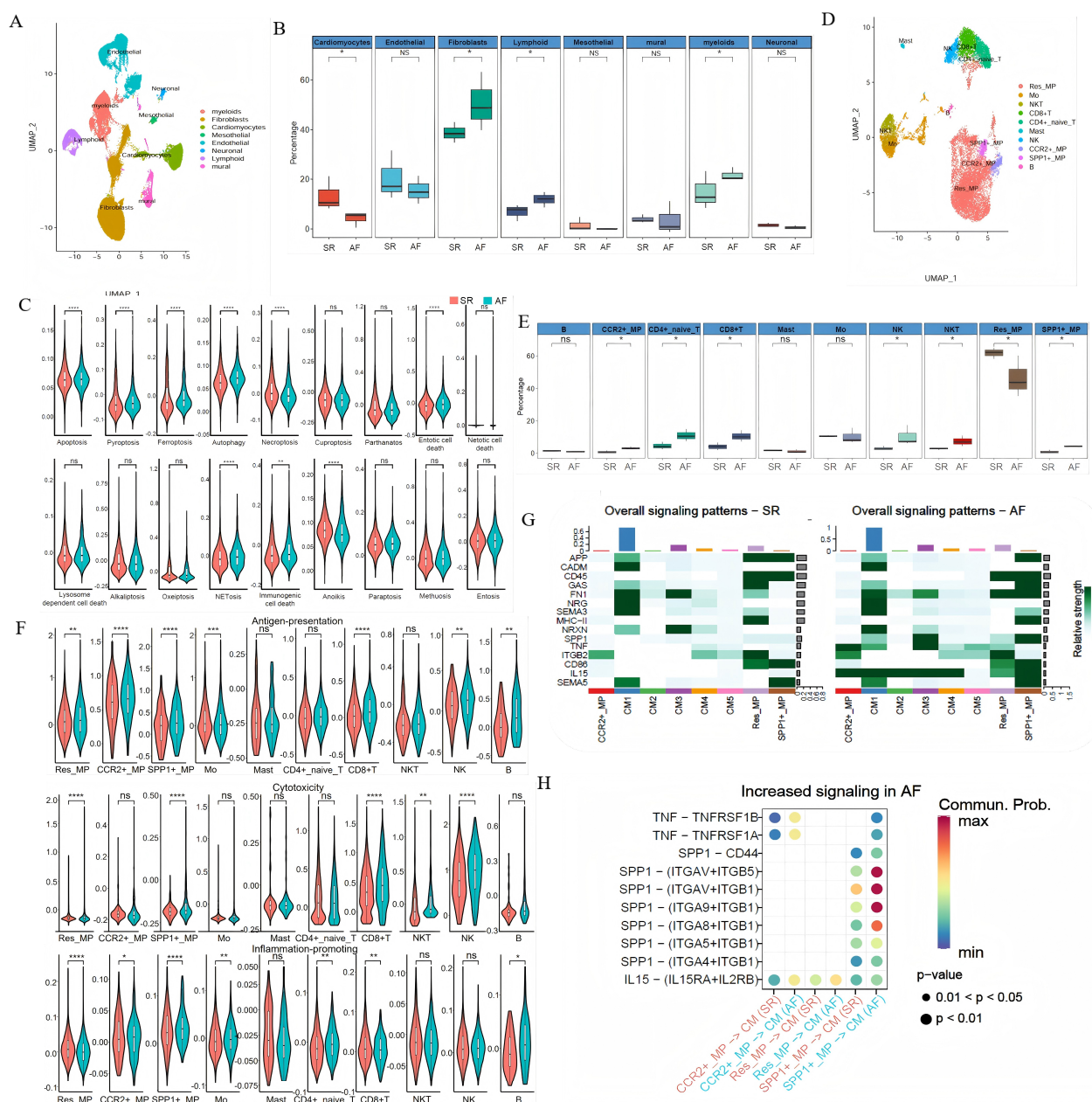


Figure 1. (A) The single-nucleus map of the right atrial tissue. (B) Subgroup proportion analysis of right atrial myocardial tissue in the samples of the SR group and the AF group ($n = 3$). (C) The violin plot shows the expression integrals of 18 types of programmed cell death genes in the right atrial cardiomyocytes of the AF group and the SR group ($n = 3$). (D) Single-nucleus map of immune cells. (E) Analysis of the proportion of immune cell subsets in the SR group and the AF group ($n = 3$). (F) The violin plot shows the expression integrals of the antigen-presenting molecule, cytotoxic molecule and pro-inflammatory cytokine gene sets in the AF group and the SR group ($n = 3$). (G) Heat map showing the relative intensity of signal patterns between macrophages and cardiomyocytes in the SR group and the AF group ($n = 3$). (H) Diagram of cell communication between macrophages and cardiomyocytes ($n = 3$). SR indicate sinus rhythm and AF, atrial fibrillation. *, **, *** and **** indicates $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$. ns means no significance. n refers to biological replicates.

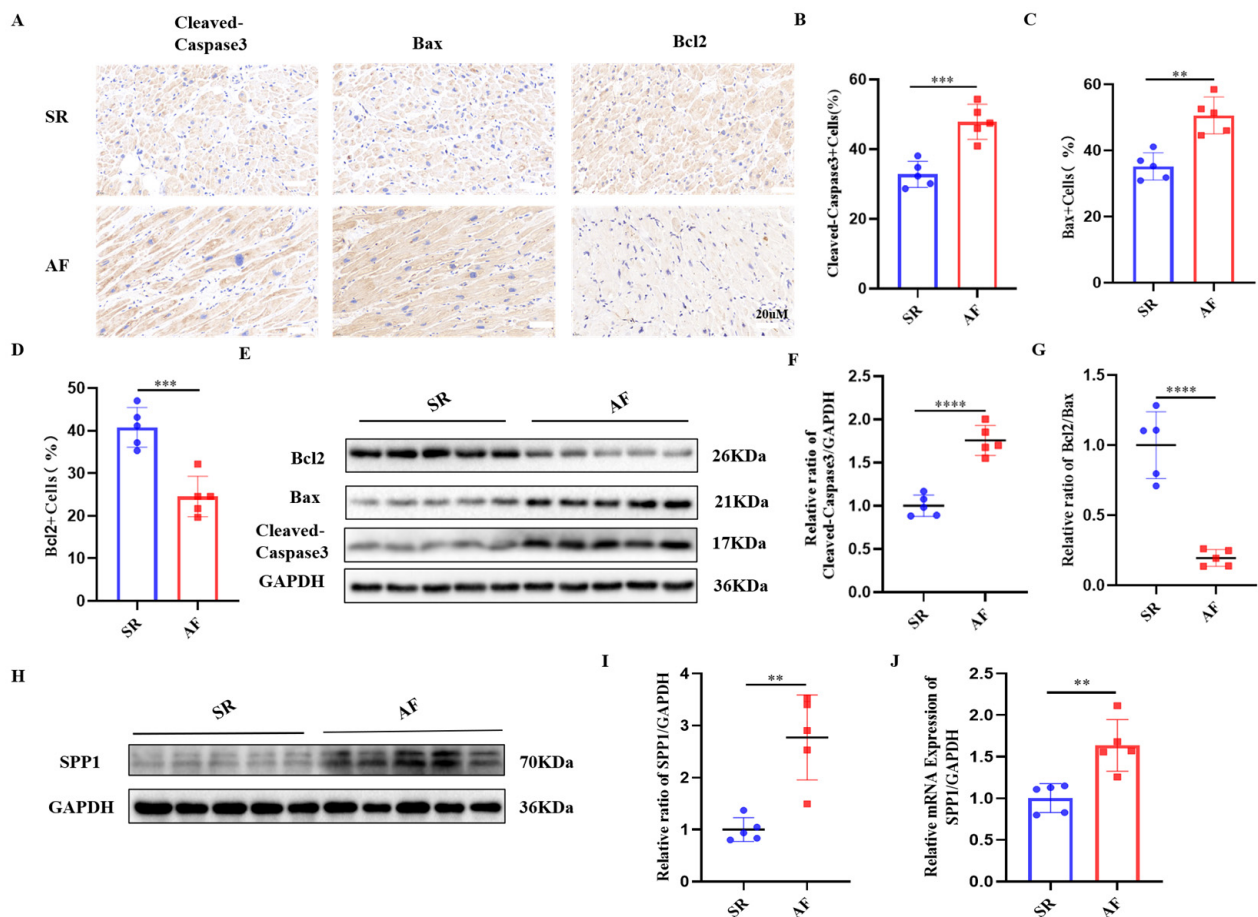


Figure 2. Expression of apoptosis-related proteins in human right atrial tissue. (A–D) Immunohistochemical staining results and statistical analysis of Cleaved-Caspase3, Bax and Bcl2 in human right atrial tissue. Scale bars, 20 μ M ($n = 5$). (E–G) The protein expression levels of cleaved caspase-3, Bax and Bcl-2 in atrial tissues from patients in the SR group and AF group were detected by Western blot ($n = 5$). (H,I) The protein expression levels of SPP1 in atrial tissues from patients in the SR group and AF group were detected by Western blot ($n = 5$). (J) The mRNA expression levels of SPP1 in atrial tissues from patients in the SR group and AF group were detected by qPCR ($n = 5$). SR indicates sinus rhythm and AF, atrial fibrillation. **, *** and **** indicates $p < 0.01$, $p < 0.001$ and $p < 0.0001$. ns means no significance. n refers to biological replicates.

3.3. SPP1⁺ Macrophages Enhance the Susceptibility to Atrial Fibrillation in Mice

To investigate the role of SPP1⁺ macrophages in AF susceptibility, we employed an AAV9 vector (HBAV2/9-F4/80-m-Spp1-3xflag-mcherry) under the control of the F4/80 promoter to selectively overexpress Spp1 in murine macrophages, thereby generating a novel mouse model of SPP1⁺ macrophage enrichment. Successful transgene expression was confirmed via Western blot and qPCR (Figure 3A–C). AAV9-SPP1 mice exhibited a significantly increased susceptibility to AF induction and prolonged AF duration compared to wild-type (WT) controls ($p < 0.05$) (Figure 3D–F). In addition, echocardiography indicated that this enhanced arrhythmogenic phenotype occurred in the absence of overt cardiac dysfunction, as left ventricular ejection fraction (LVEF) and fractional shortening (FS) remained unchanged (Figure 3G–I).

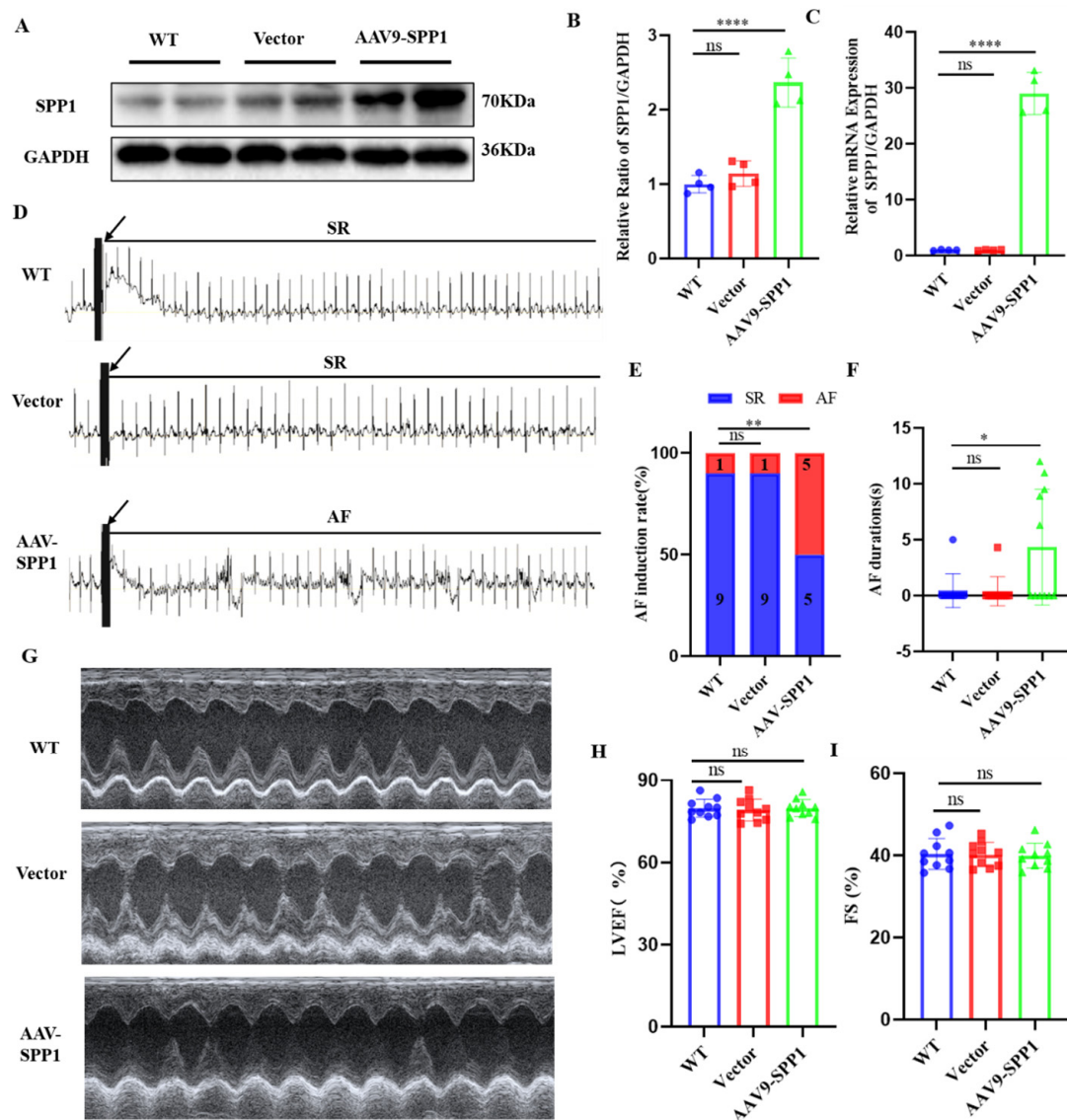


Figure 3. Mice with SPP1, specifically overexpressing macrophages, have an increased susceptibility to atrial fibrillation. (A–C) Three weeks after transfection with HBAAV2/9-F4/80-m-Spp1, the successful establishment of the mouse model with SPP1-specifically overexpressing macrophages was verified by Western blot and qPCR ($n = 4$). (D) Electrocardiogram changes before and after programmed electrical stimulation-induced AF. (E,F) The induction rate of AF and the duration of AF in WT, Vector and AAV-SPP1 mice ($n = 10$). (G–I) Echocardiographic measurement of cardiac function, statistical analysis of LVEF(H) and FS(I) in mice cardiac function ($n = 10$). *, ** and **** indicate $p < 0.05$, $p < 0.01$ and $p < 0.0001$. ns means no significance. SR indicates sinus rhythm; AF, atrial fibrillation; WT, wild-type; Vector, mice injected with negative control Adeno-associated virus serotype 9 (HBAAV2/9-F4/80-mcherry); AAV-SPP1, mice injected with Adeno-associated virus serotype 9 (HBAAV2/9-F4/80-m-Spp1-3xflag-mcherry); LVEF, left ventricular ejection fraction; FS, fractional shortening. n refers to biological replicates.

3.4. AAV9-SPP1 Mice Exhibit Apoptosis

Single-nucleus RNA sequencing (snRNA-seq) analysis identified a significant elevation in cardiomyocyte apoptosis in patients with AF compared to those with SR. This finding was further validated in a mouse model with macrophage-specific SPP1 overexpression. Following the induction of atrial fibrillation (AF) via programmed electrical stimulation (PES), heart specimens were collected, and the expression levels of apoptosis-related proteins were quantified using Western blot analysis and immunofluorescence (IF) staining.

Western blot analysis revealed a pronounced pro-apoptotic shift in the atrial tissue of AAV9-SPP1 mice compared to wild-type (WT) controls. Specifically, the protein expression of the pro-apoptotic marker Bax was significantly upregulated, while that of the anti-apoptotic protein Bcl-2 was downregulated. Consequently, the Bcl2/Bax ratio was decreased by 4.16 times. Concordantly, the expression of Cleaved-Caspase3, an executioner protease of apoptosis, was increased by 1.72 times (Figure 4A–C). Immunofluorescence (IF) staining corroborated the Western blot findings. Compared with the WT group, the atrial tissues in the AAV9-SPP1 group exhibited significantly enhanced staining intensity and a wider distribution range of Bax and Cleaved-Caspase3, while the expression level of Bcl2 was significantly decreased (Figure 4D–G). These findings suggest that macrophage-specific SPP1 overexpression induces significant cardiomyocyte apoptosis, thereby establishing a direct link between SPP1⁺ macrophages and the apoptotic pathway in atrial fibrillation.

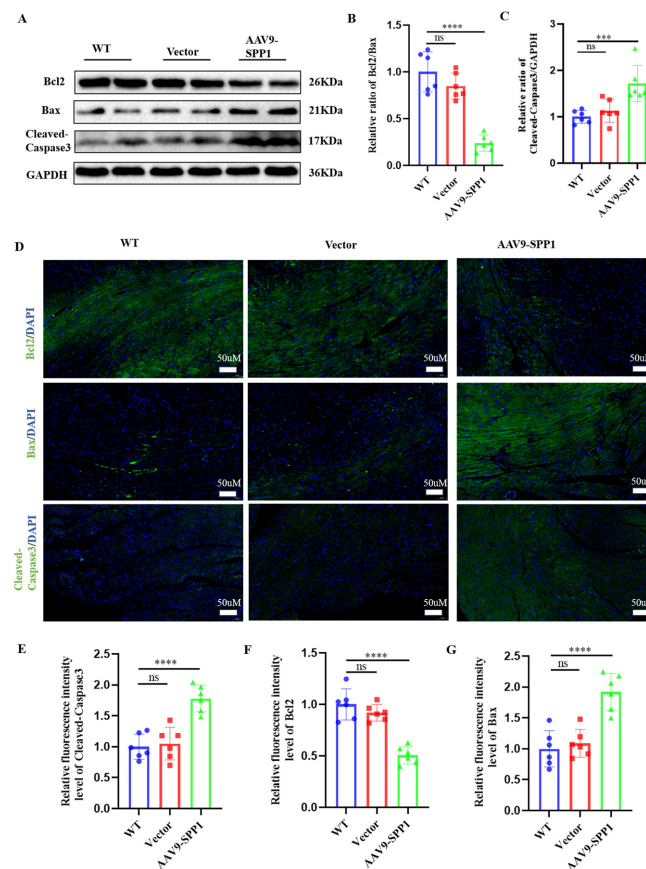


Figure 4. Expression of apoptosis-related proteins in myocardial tissues of mice with SPP1-specifically overexpressing macrophages. (A–C) The protein expression levels of Cleaved-Caspase3, Bax and Bcl2 in atrial tissues from mice in the WT group, Vector group and AAV-SPP1 group were detected by Western blot ($n = 6$). (D–G) Immunofluorescence staining and statistical analysis results of the Bcl2, Bax, Cleaved-Caspase3 (green), with nuclear staining using DAPI (blue) ($n = 6$). *** and **** indicate $p < 0.001$ and $p < 0.0001$. ns means no significance. WT indicates wild-type; Vector, mice injected with negative control Adeno-associated virus serotype 9 (HBAAV2/9-F4/80-mcherry); AAV-SPP1, mice injected with Adeno-associated virus serotype 9 (HBAAV2/9-F4/80-m-Spp1-3xflag-mcherry). n refers to biological replicates.

3.5. SPP1⁺ Macrophage Subset Induces Cardiomyocyte Apoptosis

Analysis of our snRNA-seq data suggests that SPP1⁺ macrophages potentially promote cardiomyocyte apoptosis via the SPP1 ligand-receptor signaling pathway. To further investigate this interaction, we established an SPP1-overexpressing macrophage model by transfecting RAW264.7 cells with a lentiviral vector carrying the SPP1 gene (ROE).

Cells transfected with a non-targeting control lentiviral vector (RNC) served as the control. Transfection efficiency was confirmed by fluorescence microscopy (Figure 5A), and successful SPP1 overexpression was validated at both the protein and transcript levels by Western blot analysis (Figure 5B,C) and qPCR (Figure 5D), respectively. SPP1-overexpressing macrophages were constructed in this study, and co-cultured with HL-1 cardiomyocytes to investigate their functional crosstalk. The experimental design comprised three groups: the untreated HL-1 group (HL-1), the co-culture group of HL-1 with RAW264.7 transfected with the non-targeting control lentiviral vector (HL-1+RNC), and the co-culture group of HL-1 with RAW264.7 transfected with SPP1-overexpressing lentivirus (HL-1+ROE). Following 24 h of co-cultivation, the apoptosis level of HL-1 cardiomyocytes was assessed by flow cytometry and Western blot analysis. Compared with the HL-1 group, the expression levels of apoptosis-related proteins Bax and Cleaved-Caspase3 in the HL-1+ROE group were significantly increased, while the expression level of the anti-apoptotic protein Bcl2 was significantly decreased. In contrast, there were no significant changes in the protein expression levels of Bax, Cleaved-Caspase3, and Bcl2 in the HL-1+RNC group (Figure 5E–G). Flow cytometry was performed to detect HL-1 cardiomyocyte apoptosis using Annexin V-FITC/PI double staining, a standard method for distinguishing early apoptotic, late apoptotic/necrotic, and viable cells. The results showed that compared with the HL-1 group, the total apoptotic rate (sum of early apoptotic cells [Annexin V⁺/PI[−]] and late apoptotic/necrotic cells [Annexin V⁺/PI⁺]) of HL-1 cardiomyocytes in the HL-1+ROE group was significantly increased ($4.92\% \pm 0.64\%$ vs. $1.38\% \pm 0.15\%$, $p < 0.0001$). In contrast, there was no significant difference in the total apoptotic rate, early apoptotic cell proportion, or late apoptotic/necrotic cell proportion of HL-1 cardiomyocytes between the HL-1+RNC group and the HL-1 group ($1.57\% \pm 0.11\%$ vs. $1.38\% \pm 0.15\%$, $p > 0.05$) (Figure 5H,I).

3.6. SPP1⁺ Macrophages May Induce Cardiomyocyte Apoptosis by Regulating the PI3K/AKT Signaling Pathway

To investigate the role of SPP1 signaling in cardiomyocytes, KEGG enrichment analysis of differential genes was performed on cardiomyocytes from patients with AF compared to those with SR. KEGG pathway enrichment analysis of the differentially expressed genes (DEGs) revealed significant associations with several critical pathways, most notably oxidative phosphorylation, the PI3K/AKT signaling pathway, and dilated cardiomyopathy (Figure 6A). Gene Set Enrichment Analysis (GSEA) further revealed a significant negative enrichment of the PI3K/AKT signaling pathway in the AF group compared to the SR controls (Figure 6B). Based on these findings, we propose that SPP1 signaling induces cardiomyocyte apoptosis through the inhibition and downregulation of the cardioprotective PI3K/AKT signaling pathway. To experimentally validate this hypothesis, the PI3K/AKT activator Recilisib was used for co-treatment (HL-1+ROE+Recilisib), and the levels of Cleaved-Caspase3, Bax, Bcl2, p-PI3K, and p-AKT in cardiomyocytes within the co-culture system were detected simultaneously. The results showed that compared with the HL-1 group, the HL-1+ROE group had decreased protein levels of Bcl2, p-PI3K and p-AKT, and increased levels of Cleaved-Caspase3 and Bax. Compared with the HL-1+ROE group, the HL-1+ROE+Recilisib group could reverse the changes in protein expression induced by the HL-1+ROE group (Figure 6C–G). Collectively, the addition of Recilisib, a PI3K/AKT activator, was sufficient to rescue HL-1 cells from apoptosis induced by SPP1⁺ macrophages, indicating that the suppression of PI3K/AKT pathway plays a critical and necessary role in the execution of cardiomyocyte injury under these conditions. These data demonstrate that SPP1⁺ macrophages may promote cardiomyocyte apoptosis by suppressing the PI3K/AKT signaling pathway, a key cardioprotective mechanism.

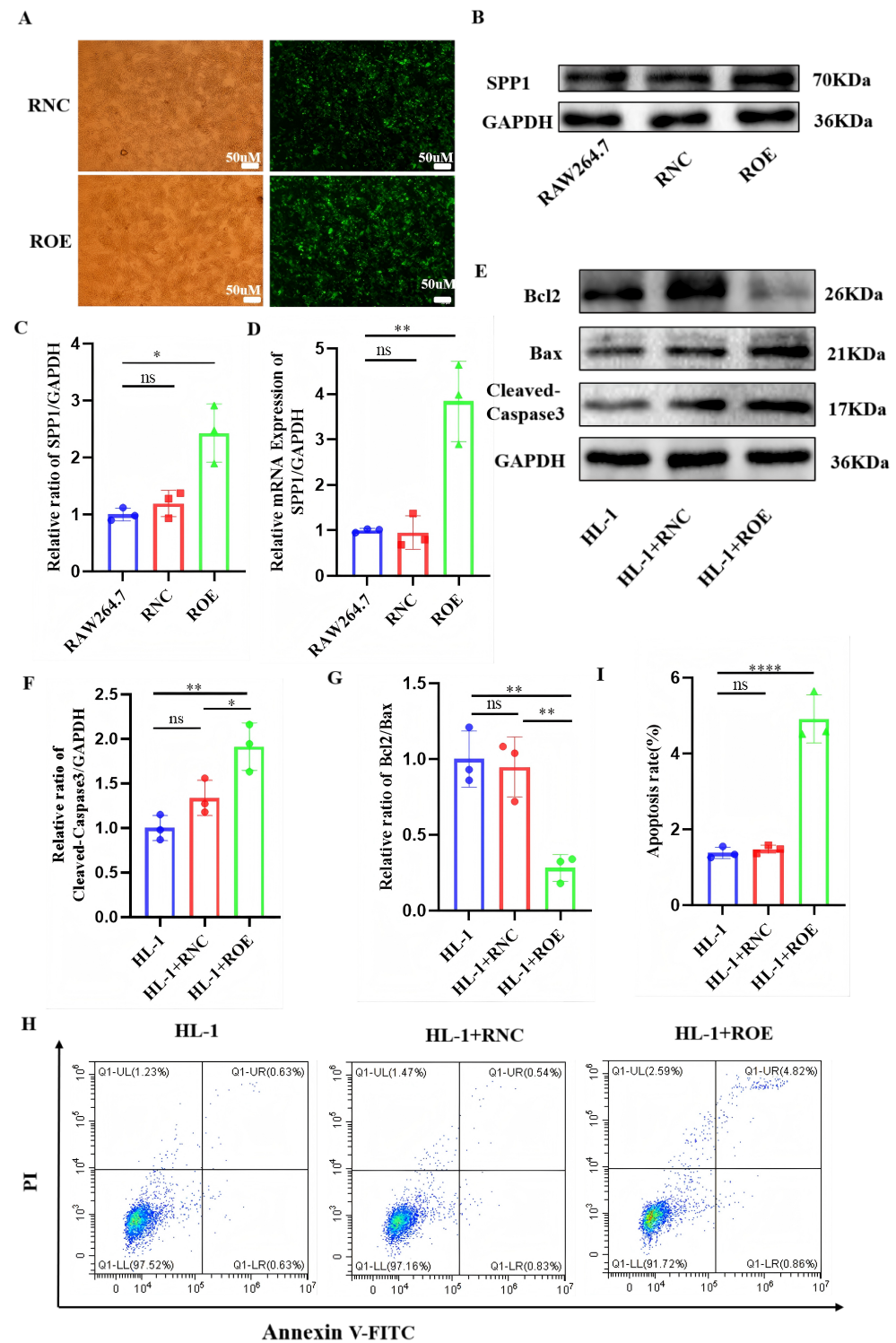


Figure 5. SPP1+ Macrophage induces cardiomyocyte apoptosis. (A–D) 72 h after RAW264.7 cells were transfected with SPP1-overexpressing lentivirus, the transfection efficiency was verified by fluorescence (A), Western blot (B,C) and qPCR (D) ($n = 3$). (E–G) After 24 h of co-culturing HL-1 cells with RAW264.7 cells, the protein expression levels of Bax and cleaved-caspase-3 increased, while the protein expression level of Bcl-2 decreased ($n = 3$). (H,I) The apoptosis level of HL-1 cells after 24 h co-culture with RAW264.7 cells was detected by flow cytometry ($n = 3$). *, ** and **** indicate $p < 0.05$, $p < 0.01$ and $p < 0.0001$. ns means no significance. RNC indicates RAW264.7 cells transfected with lentiviral vectors; RAW264.7 cells transfected with SPP1-overexpressing lentivirus; HL-1+RNC, co-culture group of HL-1 with RAW264.7 transfected with lentiviral vector; HL-1+ROE, co-culture group of HL-1 with RAW264.7 transfected with SPP1-overexpressing lentivirus. n refers to biological replicates.

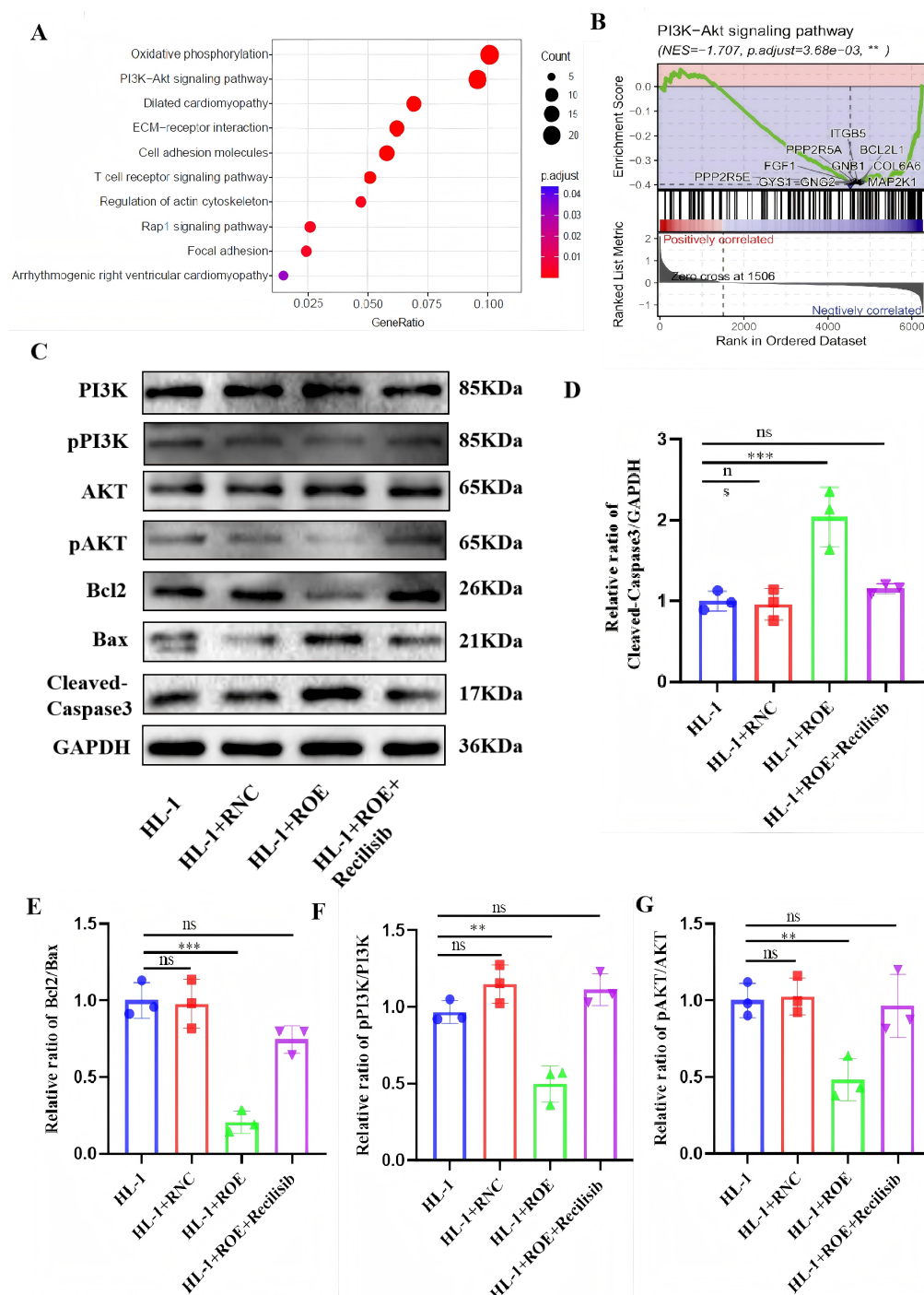


Figure 6. SPP1+ macrophages induce cardiomyocyte apoptosis by regulating the PI3K/AKT signaling pathway. (A) KEGG enrichment analysis of differential genes in human cardiomyocytes between SR and AF groups. (B) GSEA enrichment analysis showed the association of the PI3K-AKT signaling pathway in the AF group. ** indicates $p < 0.01$. (C–G) In the co-culture system, Recilisib was added for co-treatment, and the protein expression levels of Bax, Bcl2, and Cleaved-Caspase3 were detected by Western blot ($n = 3$). ** and *** indicate $p < 0.01$ and $p < 0.001$. ns means no significance. Recilisib, the activator of PI3K/AKT signaling pathway. HL-1+RNC indicates co-culture group of HL-1 with RAW264.7 transfected with lentiviral vector; HL-1+ROE, co-culture group of HL-1 with RAW264.7 transfected with SPP1-overexpressing lentivirus; HL-1+ROE+Recilisib, HL-1 cells were co-cultured with RAW264.7 cells transfected with SPP1-overexpressing lentivirus, with simultaneous co-treatment of Recilisib. n refers to biological replicates.

4. Discussion

AF remains a challenging clinical entity with high recurrence rates and limited therapeutic efficacy. This study provides compelling evidence that SPP1⁺ macrophages play a pivotal role in promoting cardiomyocyte apoptosis through suppression of the cardioprotective PI3K/AKT signaling pathway, thereby increasing AF susceptibility. Our snRNA-seq analysis revealed substantial immune cell infiltration, particularly of SPP1⁺ macrophages, within the atrial tissue of AF patients compared to SR controls. This finding aligns with emerging literature highlighting the role of inflammation and immune activation in AF pathogenesis [20–23]. The enhanced communication between SPP1⁺ macrophages and cardiomyocytes, as demonstrated by ligand-receptor interaction analysis, suggests a direct paracrine mechanism by which macrophages may influence cardiomyocyte survival.

Recent studies have highlighted that immune cell infiltration and immune remodeling are key pathological processes in the progression of atrial fibrillation (AF), with macrophages playing an especially critical role in atrial tissue remodeling during AF [24,25]. Our cell–cell communication analysis revealed intense cross-talk between SPP1⁺ macrophages and atrial cardiomyocytes, which may further amplify pro-apoptotic and pro-inflammatory responses in the atria. This aligns with previous findings by Maarten Hulsmans et al., who identified that cell–cell interaction analysis identifies SPP1 as a pleiotropic signal that promotes AF through crosstalk with local immune cells and stromal cells [14]. However, its specific effect on cardiomyocytes has not been previously reported. We further validated the pro-apoptotic role of SPP1⁺ macrophages using both in vivo and in vitro models. In a murine model with macrophage-specific SPP1 overexpression, we observed a significantly increased susceptibility to AF induction and prolonged AF duration, accompanied by significant cardiomyocyte apoptosis. Consistent with the in vivo findings, in vitro co-culture of SPP1-overexpressing macrophages with HL-1 cardiomyocytes resulted in a significantly elevated apoptotic rate, directly demonstrating the causal role of macrophage-derived SPP1 signaling in promoting cardiomyocyte death. Our study further confirms that targeting SPP1⁺ macrophages may represent a more precise strategy to intervene in AF-related immune remodeling compared with non-specific anti-inflammatory therapies. We identified the PI3K/AKT pathway as a key downstream target of SPP1 signaling. The downregulation of phosphorylated PI3K and AKT in cardiomyocytes exposed to SPP1⁺ macrophages, along with the rescue of apoptosis by the PI3K/AKT activator Recilisib, supports the notion that restoring PI3K/AKT activity is sufficient to block the apoptotic signal, positioning this pathway as a pivotal downstream node in the injury cascade initiated by SPP1⁺ macrophages. Our cell–cell communication analysis predicts a strong interaction between macrophage-derived SPP1 and integrin β 1 (ITGB1) on cardiomyocytes, providing a specific ligand–receptor axis for the observed intercellular signaling. Therefore, we hypothesize that in atrial fibrillation, SPP1⁺ macrophages act on cardiomyocyte ITGB1, inhibit the PI3K/AKT pathway, and thereby promote cardiomyocyte apoptosis. In future studies, we will validate this interaction between SPP1⁺ macrophages and cardiomyocytes through experiments such as cardiomyocyte-specific knockout of the ITGB1 gene. Moreover, our findings highlight apoptosis as a dominant form of programmed cell death in AF. We propose that this widespread cardiomyocyte loss is a fundamental mechanism underlying the characteristic structural remodeling—including atrial wall thinning and interstitial fibrosis—that creates a vulnerable substrate for the initiation and maintenance of reentrant circuits. The significant reduction in Bcl2 and increase in Bax and Cleaved-Caspase3 in both human and mouse AF models further corroborate the role of apoptosis in AF progression, which is consistent with the findings of Halil Fedai et al., who demonstrated that apoptosis plays a crucial role in the development of AF.

Our study has several limitations that warrant careful consideration when interpreting the findings. Firstly, the clinical sample size was relatively small, comprising only three patients per group (AF and SR). This small sample size may limit the statistical power to detect subtle differences in cell subset proportions or gene expression patterns, and the generalizability of our conclusions to the broader AF population. The absence of healthy controls represents a limitation in the clinical sample design of this study. Future research could explore minimally invasive approaches (such as endomyocardial biopsy) or utilize atrial tissue from patients undergoing cardiac surgery for indications other than atrial fibrillation or rheumatic heart valve disease as alternative controls to further validate our conclusions. Additionally, we aim to collaborate with more cardiac centers to obtain atrial tissues from AF patients with other etiologies (e.g., hypertension, heart failure) for comparative studies. More refined techniques, such as single-cell sequencing, will be employed to validate the macrophage signatures identified here in larger, multi-etiology cohorts in the future. Secondly, the in vitro transwell co-culture system lacks the complexity of the in vivo atrial microenvironment, including other cell types (e.g., atrial fibroblasts, endothelial cells) and extracellular matrix components. These missing elements may modulate the interaction between SPP1⁺ macrophages and cardiomyocytes, and 3D culture systems or organoid models could better simulate in vivo conditions to yield more accurate results. Finally, while we confirmed that the PI3K/AKT signaling pathway mediates SPP1⁺ macrophage-induced cardiomyocyte apoptosis, SPP1⁺ macrophages may induce apoptosis through additional pathways—for instance, by secreting miRNAs via exosomes or other pro-inflammatory cytokines to regulate specific molecules, thereby triggering cardiomyocyte apoptosis or inflammation. The existence of alternative pathways means that targeting the PI3K/AKT pathway alone may not fully abrogate the pro-apoptotic effects of SPP1⁺ macrophages, highlighting the need for further studies to explore potential crosstalk between these pathways. Despite these limitations, our core findings—linking SPP1⁺ macrophages to cardiomyocyte apoptosis via PI3K/AKT suppression—provide a novel framework for understanding AF immunopathogenesis and identify promising therapeutic targets.

5. Conclusions

In summary, single-nucleus RNA sequencing revealed a significant accumulation of SPP1⁺ macrophages in the atrial myocardium of patients with AF. We further demonstrated that these macrophages promote cardiomyocyte apoptosis, likely by the suppression of the cardioprotective PI3K/AKT signaling pathway, which establishes a direct mechanistic link to increased AF susceptibility. These findings provide novel insights into the immunometabolic mechanisms underlying AF, unveiling SPP1⁺ macrophages and the associated signaling pathway as promising therapeutic targets and potential biomarkers for the condition. This work lays a solid foundation for the development of innovative treatment strategies aimed at modulating the cardiac immune environment to prevent or reverse atrial remodeling.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jcdd13020080/s1>, Document S1: 18PCDgenes; Table S1: Summary of clinical data of the enrolled samples. SR indicate sinus rhythm; AF, atrial fibrillation; BMI, Body Mass Index; LVEF, left ventricular ejection fraction; WBC, White Blood Cell; ESR, Erythrocyte Sedimentation Rate and CRP, C-Reactive Protein; Table S2: Quality metrics of atrial tissue samples after sequencing; Figure S1: Cycle threshold (Ct) value of GAPDH.

Author Contributions: Conceptualization, J.L.; methodology, W.W. and Y.D.; Validation, L.H., L.L. and Y.J.; writing—original draft preparation, W.W., Y.D. and H.Y.; writing—review and editing, Z.X. and J.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This research has been approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University, and has been carried out in strict accordance with all ethical guidelines involving human participation (approval O-Medical Research Ethics Review [2025] NO. (171)). All applicable international, national and/or institutional guidelines for the care and use of animals were followed. This study and included experimental procedures were approved by the Animal Ethics Committee of Nanchang University (approval No. NCULAE-20250901005).

Data Availability Statement: All authors have read and approved the final manuscript. Availability of data and materials: The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Brundel, B.; Ai, X.; Hills, M.T.; Kuipers, M.F.; Lip, G.Y.H.; de Groot, N.M.S. Atrial fibrillation. *Nat. Rev. Dis. Primers* **2022**, *8*, 21. [\[CrossRef\]](#)
2. Tsao, C.W.; Aday, A.W.; Almarazooq, Z.I.; Anderson, C.A.M.; Arora, P.; Avery, C.L.; Baker-Smith, C.M.; Beaton, A.Z.; Boehme, A.K.; Buxton, A.E.; et al. Heart Disease and Stroke Statistics-2023 Update: A Report from the American Heart Association. *Circulation* **2023**, *147*, e93–e621. [\[CrossRef\]](#)
3. Benjamin, E.J.; Muntner, P.; Alonso, A.; Bittencourt, M.S.; Callaway, C.W.; Carson, A.P.; Chamberlain, A.M.; Chang, A.R.; Cheng, S.; Das, S.R.; et al. Heart Disease and Stroke Statistics-2019 Update: A Report from the American Heart Association. *Circulation* **2019**, *139*, e56–e528. Correction in *Circulation* **2020**, *141*, e33. <https://doi.org/10.1161/CIR.0000000000000746>. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Van Gelder, I.C.; Rienstra, M.; Bunting, K.V.; Casado-Arroyo, R.; Caso, V.; Crijns, H.; De Potter, T.J.R.; Dwight, J.; Guasti, L.; Hanke, T.; et al. 2024 ESC Guidelines for the management of atrial fibrillation developed in collaboration with the European Association for Cardio-Thoracic Surgery (EACTS). *Eur. Heart J.* **2024**, *45*, 3314–3414. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Camm, A.J.; Al-Khatib, S.M.; Calkins, H.; Halperin, J.L.; Kirchhof, P.; Lip, G.Y.; Nattel, S.; Ruskin, J.; Banerjee, A.; Blendea, D.; et al. A proposal for new clinical concepts in the management of atrial fibrillation. *Am. Heart J.* **2012**, *164*, 292–302.e291. [\[CrossRef\]](#)
6. Weng, L.C.; Preis, S.R.; Hulme, O.L.; Larson, M.G.; Choi, S.H.; Wang, B.; Trinquart, L.; McManus, D.D.; Staerk, L.; Lin, H.; et al. Genetic Predisposition, Clinical Risk Factor Burden, and Lifetime Risk of Atrial Fibrillation. *Circulation* **2018**, *137*, 1027–1038. [\[CrossRef\]](#)
7. Calkins, H. Important Differences Exist Between Atrial Fibrillation and Atrial Flutter in Atrial Remodeling. *J. Am. Coll. Cardiol.* **2020**, *76*, 389–390. [\[CrossRef\]](#)
8. Dobrev, D.; Heijman, J.; Hiram, R.; Li, N.; Nattel, S. Inflammatory signalling in atrial cardiomyocytes: A novel unifying principle in atrial fibrillation pathophysiology. *Nat. Rev. Cardiol.* **2023**, *20*, 145–167. [\[CrossRef\]](#)
9. Sheng, Y.; Wang, Y.Y.; Chang, Y.; Ye, D.; Wu, L.; Kang, H.; Zhang, X.; Chen, X.; Li, B.; Zhu, D.; et al. Deciphering mechanisms of cardiomyocytes and non-cardiomyocyte transformation in myocardial remodeling of permanent atrial fibrillation. *J. Adv. Res.* **2024**, *61*, 101–117. [\[CrossRef\]](#)
10. Ninni, S.; Dombrowicz, D.; de Winther, M.; Staels, B.; Montaigne, D.; Nattel, S. Genetic Factors Altering Immune Responses in Atrial Fibrillation: JACC Review Topic of the Week. *J. Am. Coll. Cardiol.* **2024**, *83*, 1163–1176. [\[CrossRef\]](#) [\[PubMed\]](#)
11. Wong, N.R.; Mohan, J.; Kopecky, B.J.; Guo, S.; Du, L.; Leid, J.; Feng, G.; Lokshina, I.; Dmytrenko, O.; Luehmann, H.; et al. Resident cardiac macrophages mediate adaptive myocardial remodeling. *Immunity* **2021**, *54*, 2072–2088.e2077. [\[CrossRef\]](#)
12. Liu, Y.; Shi, Q.; Ma, Y.; Liu, Q. The role of immune cells in atrial fibrillation. *J. Mol. Cell Cardiol.* **2018**, *123*, 198–208. [\[CrossRef\]](#)
13. Sun, Z.; Zhou, D.; Xie, X.; Wang, S.; Wang, Z.; Zhao, W.; Xu, H.; Zheng, L. Cross-talk between macrophages and atrial myocytes in atrial fibrillation. *Basic Res. Cardiol.* **2016**, *111*, 63. [\[CrossRef\]](#)
14. Hulsmans, M.; Schloss, M.J.; Lee, I.H.; Bapat, A.; Iwamoto, Y.; Vinegoni, C.; Paccalet, A.; Yamazoe, M.; Grune, J.; Pabel, S.; et al. Recruited macrophages elicit atrial fibrillation. *Science* **2023**, *381*, 231–239. [\[CrossRef\]](#)

15. Chen, Y.; Ouyang, T.; Yin, Y.; Fang, C.; Tang, C.E.; Luo, J.; Luo, F. Analysis of infiltrated immune cells in left atriums from patients with atrial fibrillation and identification of circRNA biomarkers for postoperative atrial fibrillation. *Front. Genet.* **2022**, *13*, 1003366. [[CrossRef](#)]
16. Smorodinova, N.; Blaha, M.; Melenovsky, V.; Rozsivalova, K.; Pridal, J.; Durisova, M.; Pirk, J.; Kautzner, J.; Kucera, T. Analysis of immune cell populations in atrial myocardium of patients with atrial fibrillation or sinus rhythm. *PLoS ONE* **2017**, *12*, e0172691. [[CrossRef](#)] [[PubMed](#)]
17. Zhao, Y.; Huang, Z.; Gao, L.; Ma, H.; Chang, R. Osteopontin/SPP1: A potential mediator between immune cells and vascular calcification. *Front. Immunol.* **2024**, *15*, 1395596. [[CrossRef](#)]
18. Kahles, F.; Findeisen, H.M.; Bruemmer, D. Osteopontin: A novel regulator at the cross roads of inflammation, obesity and diabetes. *Mol. Metab.* **2014**, *3*, 384–393. [[CrossRef](#)]
19. He, L.; Liu, R.; Yue, H.; Zhang, X.; Pan, X.; Sun, Y.; Shi, J.; Zhu, G.; Qin, C.; Guo, Y. Interaction between neutrophil extracellular traps and cardiomyocytes contributes to atrial fibrillation progression. *Signal Transduct. Target. Ther.* **2023**, *8*, 279. [[CrossRef](#)] [[PubMed](#)]
20. Huang, M.; Huiskes, F.G.; de Groot, N.M.S.; Brundel, B. The Role of Immune Cells Driving Electropathology and Atrial Fibrillation. *Cells* **2024**, *13*, 311. [[CrossRef](#)] [[PubMed](#)]
21. Chen, X.; Yu, L.; Meng, S.; Zhao, J.; Huang, X.; Wang, Z.; Zhou, Z.; Huang, Y.; Hong, T.; Duan, J.; et al. Inhibition of TREM-1 ameliorates angiotensin II-induced atrial fibrillation by attenuating macrophage infiltration and inflammation through the PI3K/AKT/FoxO3a signaling pathway. *Cell. Signal.* **2024**, *124*, 111458. [[CrossRef](#)]
22. Keefe, J.A.; Wang, J.; Song, J.; Ni, L.; Wehrens, X.H.T. Immune cells and arrhythmias. *Cardiovasc. Res.* **2025**, *121*, 382–395. [[CrossRef](#)] [[PubMed](#)]
23. Hutschalik, T.; Ozgul, O.; Casini, M.; Szabo, B.; Peyronnet, R.; Bartulos, O.; Argenziano, M.; Schotten, U.; Matsa, E. Immune response caused by M1 macrophages elicits atrial fibrillation-like phenotypes in coculture model with isogenic hiPSC-derived cardiomyocytes. *Stem Cell Res. Ther.* **2024**, *15*, 280. [[CrossRef](#)] [[PubMed](#)]
24. Huynh, K. Novel macrophage targets for the treatment of atrial fibrillation. *Nat. Rev. Cardiol.* **2023**, *20*, 648. [[CrossRef](#)] [[PubMed](#)]
25. Yao, Y.; Yang, M.; Liu, D.; Zhao, Q. Immune remodeling and atrial fibrillation. *Front. Physiol.* **2022**, *13*, 927221. [[CrossRef](#)]

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