2. Methods

2.1 Data collection and study cohorts for acute aortic dissection research

We collected data from patients with acute AD who were admitted to the Department of Vascular Surgery and the Department of Cardiovascular Surgery at the Second Qilu Hospital of Shandong University between January 2021 and June 2021. The diagnosis of acute AD was confirmed through aortic computed tomography angiography (CTA). CTA imaging data were processed and measured using the GE Volumeshare 7 (AW4.7) system[1]. A total of 108 patients were included as the AD group for the clinical research section, and data from 30 age- and sex-matched volunteers served as the control group (**Supplementary Table S1**). A subgroup of 20 aortic dissection tissue samples from these patients was selected for the experimental group in the basic research section, while 10 organ donors' data and aortic tissue served as the control group for the basic research section (**Supplementary Table S2**). Relevant data were collected, and adverse outcomes were defined according to related literature[2, 3]. Exclusion Criteria: Chronic aortic dissection, Intramural hematoma, Aortic ulcer, Traumatic aortic dissection, Aortic stenosis, Aortic aneurysm, History of prior aortic dissection, History of cardiac valve or coronary artery surgery, Autoimmune diseases and connective tissue disorders (such as Marfan syndrome, systemic lupus erythematosus) complicated by aortic dissection, Cases of aortic dissection during pregnancy.

2.2 Single-cell transcriptomics: sample preparation and analysis

Tissue samples (**Supplementary Table S3**) were prepared from the dissection site of aortic dissections (n=2) and normal aortic tissue (n=1), then processed using the GEXSCOPE system (Singleron, China) to achieve a 1×10^5 cells/mL suspension. Viability (>80%) was

confirmed by Trypan Blue staining (1450013, Bio-Rad, USA). Libraries were constructed using the GEXSCOPE Single-Cell Transcriptome Library Kit (Singleron GEXSCOPE, China), diluted to 4 nM and sequenced on the Illumina HiSeq X platform (2×150 bp)[4]. Raw scRNA-seq data were processed to generate gene expression matrices. Quality control was performed using FastQC v0.11.4, and adapters were trimmed using Cutadapt. Reads were aligned to GRCh38 using STAR v2.5.3a, and gene and UMI counts were quantified using featureCounts v1.6.2. Quality filtering removed cells with UMIs<30,000, genes<200 or >5,000, or mitochondrial expression>50%. Data normalization and correction were conducted using Seurat v2.3, applying NormalizeData and ScaleData to adjust for unwanted variability. The most significant 2,000 genes, as identified by FindVariableFeatures, were used for principal component analysis. Cells were clustered using FindClusters, and batch effects were adjusted using Harmony. The UMAP algorithm visualized the single-cell data in two-dimensional space, and cell types were annotated based on gene expression markers and literature. Lineage analysis used Monocle2 for skeleton construction, trajectory reconstruction, and visualization via plot_cell_trajectory [5]. Cell markers were visualized via Seurat's DoHeatmap/DotPlot. We used the Seurat FindMarkers function based on Wilcox likelihood-ratio test with default parameters (Bonferroni-corrected P values<0.05), and selected the genes expressed in more than 10% of the cells in a cluster and with an average log (Fold Change) value greater than 0.25 as differentially expressed genes (DEGs). The y-axis of volcano plot represents |pct1-pct2|, where pct1 and pct2 are the proportions of cells expressing the gene in the experimental and control groups, respectively. The VennDiagram R package helped identify genes common to two data subsets: DEGs between normal aortic and

aortic dissection tissues, and DEGs between macrophage subclusters a and b-f. DEGs underwent GO analysis via clusterProfiler.

2.3 Bioinformatics Analysis

We employed the STRING database to explore interactions within DEGs from macrophage subcluster a, identifying key genes pivotal to our study. For our study, human gene expression data (GSE153434， GSE147028, and GSE52093) and mouse data (GSE138484, GSE138558, GSE215935, and GSE222382) were retrieved from the GEO database. We identified DEGs between AD and normal tissues, setting the threshold for DEG selection at an absolute log fold change (logFC) ≥ 1 and a *P*-value < 0.05. Utilizing Venn diagrams, we integrated DEGs from single-cell transcriptomic analysis of macrophages cluster a with those from the GEO datasets to pinpoint hub genes.

2.4 Flow cytometry analysis of primary cells from human AD tissues

Human AD tissues were cut into 1-mm^3-sized pieces within 4 hours of removal. Tissue pieces were incubated with shaking at 37°C in a solution containing collagenase IV (1 mg/ml; Sigma-Aldrich). After filtering undigested tissue debris, cells were resuspended using primary cell culture medium. Cells were fixed and washed with permeabilization buffer. The cells were analyzed by flow cytometry using APC/Cyanine7 anti-human CD68 Antibody (333821, BioLegend), PE Anti-Human IL-6 Antibody (E-AB-F1206D, Elabscience), SPHK1 Recombinant Rabbit mAb (bsm-63216R, Bioss), and Goat Anti-Rabbit IgG H&L, APC conjugated (bs-0295G-APC, Bioss).

2.5 Induction of the mouse AD model

Male 3 weeks old C57BL/6J mice (Beijing Vital River Laboratory Animal Technology Co., Ltd) were housed at the animal center of The Second Qilu Hospital of Shandong University with ad libitum access to food and water. The AD model was induced with β-aminopropionitrile (BAPN), which disrupts elastin and collagen cross-linking, compromises aortic wall integrity, and promotes dissection; this approach is most effective when administered during rapid developmental stages. Prior to establishing the AD model, we treated wild-type (WT) mice with saline or PF-543 to assess toxicity. We did not see any obvious side effects on blood pressure, heart rate, or tissue damage (**Supplementary Figure S1A-C**). After conducting toxicity testing on WT mice, we used PF-543 to block the function of SPHK1 in a male mouse model of AD induced by BAPN. The mice were randomly divided into three groups: 1) control group; 2) BAPN group; and 3) BAPN + PF-543 group (n=20 per group). Mice in the BAPN group and the BAPN + PF-543 group received drinking water containing dissolved BAPN (1 g/kg/day, HY-Y1750, MCE, USA) for 28 days; the control group received normal drinking water. Starting from the age of three weeks, mice in the BAPN + PF-543 group were also administered intraperitoneal injections of the SPHK1 inhibitor PF-543 (10 mg/kg, HY-15425A, MCE, USA), while the control and BAPN groups received equivalent volumes of saline. If a mouse reached a predefined humane endpoint during the induction period, it was euthanized and an immediate necropsy was performed to confirm model induction and the presence of aortic rupture; survival time was recorded.

2.6 Aortic ultrasound examination

Mice were anesthetized using 1% isoflurane (inhalation) delivered through a precision vaporizer. The dosage was carefully adjusted to maintain a stable anesthetic plane, ensuring

the absence of pain reflexes while avoiding over-sedation. The isoflurane was administered continuously throughout the imaging procedure, and the animals' vital signs were closely monitored to ensure their well-being. This method of anesthesia was selected due to its rapid onset and short recovery time, minimizing stress on the animals. A high-frequency ultrasound imaging system for small animals (Vevo 3100LT, FUJIFILM Visualsonics, Japan) was used with a probe placed at the upper sternal acoustic window, operating at a frequency of 30 MHz. Longitudinal images of the ascending aorta, aortic arch, and its branches were obtained.

2.7 Aortic tissue dissection

28 days after induction, mice were euthanized. Euthanasia was performed using a combination of isoflurane inhalation followed by an intra-peritoneal injection of pentobarbital sodium (100 mg/kg)[6, 7]. Under a stereomicroscope, a meticulous dissection of the aorta from the heart to the iliac artery bifurcation was performed. The collected tissue samples were placed in 4% paraformaldehyde or liquid nitrogen.

2.8 Tissue section preparation and staining protocols

Tissues were encased in paraffin, and subsequently cut into slices measuring 4 μm in thickness. Aorta pathology was evaluated using Hematoxylin and eosin (H&E) staining. EvG staining (G1042, Servicebio, China) was performed to visualize collagen and elastic fibers [8]. Elastic fibers were graded based on their integrity: Grade 0 (intact fibers with normal curvature), Grade 1 (stretched fibers with loss of curvature), Grade 2 (presence of some fragmented fibers), and Grade 3 (severely damaged fibers).

2.9 Cell culture and *in vitro* intervention protocol

RAW264.7 cells (Shanghai QiDa Biotechnology Co., LTD) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (C11995500BT, Gibco, USA). *In vitro* SPHK1 function intervention: cells were seeded in 6-well plates at approximately 3×10^5 cells per well. They were randomly divided into three groups: a control group, a PF-543 (10 μM) inhibitor group[9], and an MHP (30 μM) agonist group. After 24 hours of treatment with the respective compounds, cells and supernatants were collected for further analysis.

2.10 Cell treatment

RAW264.7 cells were seeded in a 6-well plate at 3×10^5/well. We diluted 100 nM of siRNAs (SPHK1-si1, SPHK1-si2, SPHK1-si3, S1PR2-si1, S1PR2-si2) or scrambled control siRNA in 250 μL Opti-MEM medium. Separately, 5 μL of Lipofectamine RNAiMAX (13778030, Thermo Fisher Scientific Inc. Shanghai, China) was diluted in 250 μL Opti-MEM medium. These complexes were then added to each well and mixed gently. To conduct the rescue experiments, 2,000 ng of SPHK1-si3-resistant pCDH-SPHK1 expression vectors (HonorGene, China) were used for transfection in the SPHK1-si3 group. In the sphingosine-1-phosphate (S1P) supplementation group, RAW264.7 cells were incubated for 24 h with S1P (1 μM, 860552P, Sigma) prior to protein harvesting. For SPHK1 overexpression, the pCDH-SPHK1 vector was constructed by HonorGene.

2.11 Immunohistochemistry and immunofluorescence

Aortic dissection or healthy aorta tissue slides of human and mice were fixed in FFPE. Primary antibodies were applied: CD68 (1:50, A13286, ABclonal, China), SPHK1 (1:100,

10670-1-AP, Proteintech, China), MMP2 (1:200, A6247, ABclonal, China), and MMP9 (1:100, A0289, ABclonal, China), followed by a secondary antibody. Immunoreactivity scores were determined by multiplying the score[10] for staining intensity (0: no staining, 1: weakly positive, 2: moderately positive, 3: strongly positive) by the percentage of positive cells (0-100%). Representative images were collected using K-Viewer.

Sections were incubated with 50 μL of primary antibodies (CD68, 1:50, A13286, ABclonal, China; SPHK1, 1:50, 10670-1-AP, Proteintech, China; MMP2, 1:100, 66366-1-Ig, Proteintech, China; MMP9, 1:100, 222462, Zenbio, China; IL-6, 66146-1-Ig, Proteintech, China; IL-1β, 1:100, 222462, Zenbio, China). followed by a secondary antibody (FITC Goat, 1:100, AS011, ABclonal, China; Cy3 Goat, 1:100, AS008, ABclonal, China) at 50 μL per section. Representative images were collected using an automated fluorescence microscope (Axio Observer 7, ZEISS, Germany).

2.12 RNA extraction and quantitative PCR methodology

RNA was extracted from frozen sections of aortic tissues (50 mg from humans and 10 mg from mice) and cell pellets using Trizol reagent (15596026, Invitrogen, USA). For cDNA synthesis, RNA was reverse transcribed using Hifair® III 1st Strand cDNA Synthesis SuperMix (gDNA digester plus, 11141ES60, Yeasen, China. The qPCR was performed using Hieff® qPCR SYBR Green Master Mix (Low Rox Plus, 11202ES08, Yeasen, USA). Primers were listed in **Supplementary Table S4**.

2.13 Protein extraction and western blot analysis

Total protein was extracted from aortic tissues (100 mg from humans and 10 mg from mice)

using the Total Protein Extraction Kit (SW103-02, Seven, China). Primary antibodies were applied SPHK1 (1:1000, 10670-1-AP, Proteintech, China), MMP2 (1:1000, A6247, ABclonal, China), MMP9 (1:1000, A0289, ABclonal, China), S1PR2 (1:1000, 21180-1-AP, Proteintech, China), S1PR3 (1:700, A1404, ABclonal, China), IL-6 (1:700, A0286, ABclonal, China), β-Actin (1:1000, #4970, CST, USA), RhoA (1:1000, 10749-1-AP, Proteintech, China), ROCK1 (1:5000, 21850-1-AP, Proteintech, China), GAPDH (1:50000, 60004-1-Ig, Proteintech, China), HIF-1α (1:4000, A26889, ABclonal, China) and Tubulin (1:10000, A12289, ABclonal, China) mAb followed by a secondary antibody. Densitometric analysis was performed using ImageJ software (1.8.0).

2.14 Mouse peripheral blood and aortic tissue analysis via ELISA

Levels of interleukin-6 (IL-6) and interleukin-1 beta (IL-1β) in the mouse peripheral blood were measured using the Mouse IL-6 Precoated ELISA Kit (1210602, Dakewe, China), Mouse IL-1β Precoated ELISA Kit (1210122, Dakewe, China) according to the manufacturer's protocols. S1P levels in mouse aortic tissue and plasma were measured using the Mouse S1P Precoated ELISA Kit (MO-P9S425X, Meiao, China).

2.15 Statistical analysis methods

Statistical analyses employed Wilcoxon rank-sum for cell distribution, DEGs comparisons between groups or cell types, and non-normally distributed variables. Clinic categorical data used continuity-corrected Pearson χ^2 test. Independent *t*-test assessed group differences. Correlations used Pearson (parametric) or Spearman (non-parametric). Survival differences were determined via log-rank test and Kaplan-Meier curves. All statistical procedures were

performed using the SPSS software package (IBM SPSS Statistics 25). A *p*-value of less than 0.05 was considered statistically significant[11].

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