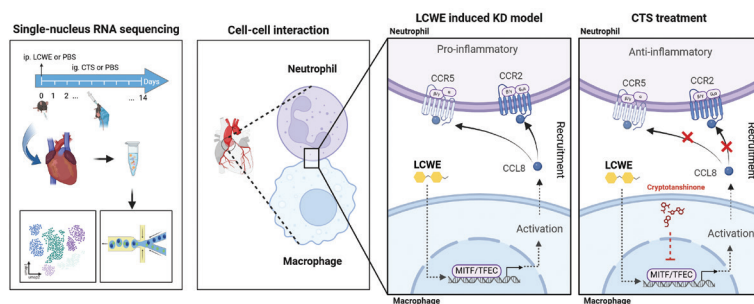


Cryptotanshinone alleviates vasculitis in Kawasaki disease by modulating macrophage-neutrophil interactions

Graphical abstract



Highlights

- Cryptotanshinone alleviates vasculitis in the LCWE-induced Kawasaki disease mouse model by modulating macrophage activation.
- The downregulation of MITF/TFEC transcription factor activity by cryptotanshinone decreases the transcription of the *Ccl8* chemokine gene.
- Limiting neutrophil recruitment prevents the further expansion of inflammation in the aortic root, thereby improving disease outcomes.

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In-brief

Through snRNA-seq, we identified a potential mechanism through which CTS alleviates vasculitis in a LCWE-induced KD mouse model. CTS modulates the activation state of macrophages by downregulating MITF/TFEC transcription factor activity, decreasing transcription of the *Ccl8* chemokine gene, and limiting the recruitment of pro-inflammatory neutrophils. This process prevents further expansion of inflammation in the aortic root and improves disease outcomes.

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Cryptotanshinone alleviates vasculitis in Kawasaki disease by modulating macrophage-neutrophil interactions

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ABSTRACT

Kawasaki disease (KD) is a form of vasculitis that affects primarily children and can lead to severe cardiovascular complications. Because current treatment options are often ineffective for some patients, new therapeutic strategies are needed. Cryptotanshinone (CTS), a compound derived from *Salvia miltiorrhiza* Bunge, has shown potential as an anti-inflammatory agent. Herein, in a mouse model of KD induced by *Lactobacillus casei* cell wall extract (LCWE), CTS was found to significantly decrease inflammation in the aortic root and coronary arteries. This treatment inhibited the activation of macrophages and neutrophils, which are critical contributors to KD. Network pharmacology analysis suggested that CTS modulates the chemokine signaling pathway, thereby inhibiting the recruitment of inflammatory cells and preventing further vasculitis progression. Single-nucleus RNA sequencing (snRNA-seq) revealed that CTS decreased macrophage numbers and chemokine activity, particularly that of Ccl8, and consequently neutrophil recruitment. Our findings suggest that CTS might provide a promising therapeutic option for KD by modulating immune cell interactions and inflammation.

Keywords: Kawasaki Disease, Vasculitis, Cryptotanshinone, Macrophages, Chemotaxis

1. INTRODUCTION

Kawasaki disease (KD) manifests primarily as coronary artery abnormalities and particularly affects children. Although KD is a self-limiting condition, untreated cases can result in severe cardiovascular complications [1]. Despite advancements in the understanding of KD pathogenesis in recent years, research examining its precise etiology and therapeutic strategies remains ongoing [2-6]. The pathogenesis of KD has been suggested to be closely associated with abnormal immune activation, particularly involving innate immune cells such as monocytes, macrophages, and neutrophils [6-10]. Notably, neutrophil infiltration into the arterial wall occurs within 2 weeks of KD onset and leads to necrotizing arteritis [9]. Nevertheless, the factors driving this intense inflammatory infiltration remain unclear.

The standard treatment for KD currently includes aspirin and intravenous immunoglobulin (IVIG), both of which significantly decrease the risk of coronary artery abnormalities. However, some patients exhibit resistance to these treatments [4], thus indicating unmet therapeutic needs. Active compounds from traditional Chinese medicine, including triptolide, quercetin, and ligustrazine, have been proposed as alternative or complementary treatments for KD [11-13]. These natural compounds are widely available and have high safety [14]. Herein, we focused on CTS, a major active component of the traditional Chinese medicine herb *Salvia miltiorrhiza* [15], which is known for its anti-inflammatory, antioxidant, and antimicrobial properties. Cryptotanshinone (CTS) has been shown to be effective against various inflammatory diseases, such as ulcerative colitis [16], gestational diabetes mellitus [17], and

cardiovascular conditions, including atherosclerosis [18] and myocardial infarction [19]. However, its role in the treatment of KD remains unexplored.

The development of network pharmacology and snRNA-seq techniques has provided new insights into understanding the pathogenesis of KD and identifying potential therapeutic targets [20, 21]. These methods facilitate the identification of inflammation-related signaling pathways and key molecules involved in disease progression, and have potential to illuminate the molecular mechanisms through which CTS might affect KD treatment. This study was aimed at investigating the therapeutic effects of CTS in a KD model, as well as its underlying mechanisms, particularly in modulating the chemotactic signaling pathways of inflammatory cells. In a mouse model of KD, we used network pharmacology and single-nucleus RNA sequencing (snRNA-seq) to evaluate the effects of CTS on vascular inflammation and myocardial damage, to elucidate how CTS might alleviate inflammation by modulating the interactions between macrophages and neutrophils. Our findings provide a novel perspective for KD treatment and establish a foundation for future clinical applications.

2. METHODS

2.1 Regents

Lactobacillus casei ATCC 334 (cat. no. BNCC186562) was purchased from Beijing Beina Chuanglian Biotechnology Co., Ltd. CTS (cat. no. HY-N0174) was obtained from MedChemExpress, USA. A 4% general-purpose paraformaldehyde solution (cat. no. BL539A) was obtained from Beijing Lange Ke Technology Co., Ltd. Hematoxylin (cat. no. G1080) and eosin (cat. no. G1100) were both purchased from Beijing Solarbio Technology Co., Ltd.

2.2 Construction of an LCWE-induced KD mouse model

Mice were purchased from GemPharmatech (animal license number SCXK 2019-0056, Nanjing, China). The mice were housed in standard laboratory conditions, under a normal temperature of 22–24°C with a 12-hour light/dark cycle.

The extraction of *Lactobacillus casei* cell wall components was conducted as previously described [22]. The strain was expanded in MRS medium (HuanKai Microbial, cat. no. 027312) at 37°C, 220 rpm, then lysed with SDS and washed with PBS. Ultrasonication for 2 hours and centrifugation yielded *Lactobacillus casei* cell wall extract (LCWE), which was subsequently quantified. Male C57BL/6 mice 4–5 weeks old were divided into a normal group (n=4), LCWE model group (n=7), low-dose CTS group (CTS-L, n=6), and high-dose CTS group (CTS-H, n=6). A single intraperitoneal injection of 1 mg LCWE or PBS was administered. The next day, the CTS-L and CTS-H groups received daily doses of 20 mg/kg and 40 mg/kg CTS, respectively, for 14 days, whereas the normal and LCWE groups received PBS.

2.3 Histological analysis

Heart tissue was fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned at 5 µm. Hematoxylin and eosin staining was performed to assess myocardial and vascular inflammation. For immunohistochemistry, mouse heart tissue paraffin sections were deparaffinized, rehydrated, subjected to antibody retrieval with sodium citrate, blocked, stained with anti-F4/80 antibody (Cell Signaling Technology, cat. no. 30325T) at a dilution of 1:100, and incubated overnight at 4°C. After three washes with 1× PBS, the slides were developed with a DAB kit (Proteintech) according to the manufacturer's protocol.

To investigate the interaction between macrophages and neutrophils, we performed immunofluorescence staining to label macrophages (F4/80), neutrophils (MPO), and neutrophil signaling receptors (CCR2 and CCR5). Furthermore, to confirm whether MITF and TFEC might form a dimer that enhances transcriptional activity, we conducted additional immunofluorescence staining to label macrophages (F4/80) and the MITF and TFEC proteins. For immunofluorescence, mouse heart tissue paraffin sections were deparaffinized, rehydrated, subjected to antibody retrieval with sodium citrate, blocked with 3% goat serum, and incubated with primary antibodies overnight at 4°C. Anti-CCR2 (Proteintech, catalog no. 16153-1-AP, 1:200), anti-CCR5 (ABclonal, catalog no. A20261, 1:300) antibodies, anti-MITF (Abcam, catalog no. ab303530, 1:500), and anti-TFEC (Proteintech, catalog no. 13547-1-AP, 1:500) were incubated with secondary antibodies at room temperature for 1 hour, then labeled with TRYG90 fluorescent dye (Guduo Biotechnology Co., Ltd., catalog no. DDS0034-1). Anti-F4/80 was detected with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibodies (1:500; Invitrogen, catalog no. A-11008), whereas anti-MPO (Santa Cruz, catalog no. sc-51741, 1:200) was detected with Alexa Fluor 594-conjugated goat anti-mouse IgG (H+L) cross-adsorbed secondary antibodies (1:500; Invitrogen, catalog no. A-11005). Incubations were conducted at room temperature for 2 hours in the dark, and nuclei were counterstained with DAPI. Images were acquired with an Olympus VS200 instrument.

2.4 Cell culture

THP-1 cells were obtained from ATCC and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. THP1 cells were pretreated with 100 ng/mL PMA for 24 h. To investigate the inhibitory effect of CTS on LCWE-induced inflammation, we first incubated the cells with LCWE at concentrations of 2.5, 5, 7.5, and 10 µg/mL. Subsequently, to cells exposed to an LCWE concentration of 2.5 µg/mL, 40 µM CTS was added, and its pharmacological effects were evaluated. To validate the role of the MITF transcription factor, we introduced 20 µM TT012 (MedChemExpress, catalog no. 1164471-33-3), a specific inhibitor of MITF transcriptional activity, under the same 2.5 µg/mL LCWE condition to determine

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whether inhibiting MITF transcriptional activity might suppress the expression of downstream transcription factors in macrophages.

2.5 Real-time qPCR

Total RNA was extracted from THP-1 cells with TRIzol reagent (Takara Bio, Japan). RNA (1,000 ng) was subjected to reverse transcription to cDNA with Hiscript II Q RT SuperMix for qPCR (catalog no. R223, Vazyme Biotech Co., Ltd., China). Quantitative RT-PCR was performed with Taq Pro Universal SYBR qPCR Master Mix (catalog no. Q712, Vazyme Biotech Co., Ltd., China) on a CFX 100 cyclor (Bio-Rad, Hercules, CA). The primers were as follows: *IL1B* forward: ATGATGGCTTATTACAGTGGCAA, reverse: GTCGGAGATTCGTAGCTGGA; *TNF* forward: GAGGCCAAGCCCTGGTATG, reverse: CGGGCCGATTGATCTCAGC; *PTGS2* forward: CAACAGAGTATGCGATGTGCTT, reverse: CCTATCAGTATTAGCCTGCTTGTC; *IL-6* forward: CCTGAACCTTCCAAAGATGGC, reverse: TTCACCAGGCAAGTCTCTCTCA; *CCL8* forward: TGGAGAGCTACACAAGAATCACC, reverse: TGGTCCAGATGCTTCATGGAA; *CCL5* forward: TCATTGCTACTGCCCTCTGC, reverse: GTTGATGTACTCCCGAACCCTCA; *CCL2* forward: CAGCCAGATGCAATCAATGCC, reverse: TGGAAATCCTGAACCCACTCT. Gene expression was calculated as $RQ=2^{-\Delta\Delta Ct}$ with normalization to GAPDH expression.

2.6 Network pharmacology

CTS-associated targets were retrieved from TCMSP (<http://tcmspnw.com/>) and SwissTargetPrediction (<http://swisstargetprediction.ch/>), whereas KD-associated targets were obtained from OMIM (<https://omim.org/>), DisGeNET (<https://www.disgenet.org/>), and GeneCards (<https://www.genecards.org/>). After deduplication, target networks were constructed for CTS and KD. The intersection yielded 45 common genes, which were subjected to Disease Ontology (DO), Gene Ontology (GO), and KEGG pathway analyses.

2.7 Data collection and integration

GSE235994 RNA-seq data from LCWE model mouse heart tissue, including a PBS group (n=5) and LCWE group (n=6), were downloaded. The data were processed and normalized with the DESeq2 package, with a focus on expression matrices of chemotactic and inflammatory genes.

GSE168732 single-cell RNA sequencing (scRNA-seq) data from human peripheral blood mononuclear cells (PBMCs), including a healthy group (n=3) and KD group (n=6, pre-IVIG treatment), were also downloaded. Monocytes were extracted for analysis, and integration with mouse heart snRNA-seq data was performed for pseudotime analysis.

GSE178799 spatial transcriptomics data from LCWE model mouse hearts, including a PBS group (n=1) and LCWE group (n=1), were integrated. Spatial location and transcriptional expression data were visualized with the ggplot2 package.

2.8 snRNA-seq library construction

Mouse heart tissue (~100 mg) was minced and dispersed in 5 mL lysis buffer, then processed with a Dounce homogenizer to release nuclei. The homogenate was filtered (70 μ m, then 40 μ m) and centrifuged at 1,000 g for 5 min at 4°C. Nuclei were resuspended in 2 mL sucrose buffer, layered over 4 mL sucrose buffer, and centrifuged again. The pellet was washed with 1 mL, then resuspended in, nuclei storage buffer. The single-nuclei suspension was used to construct the snRNA-seq library, according to the 10x Genomics Single Cell 3' Reagent Kit v3 protocol.

2.9 snRNA-seq data processing and analysis

Data were processed in Cell Ranger software (v3.1.0, 10x Genomics) and analyzed with Seurat to remove low-quality cells. Data integration, normalization, clustering, and dimensionality reduction were performed with the Seurat package (v5.0.1) in R (v4.3.3). DoubletFinder was used to remove doublet interference, and cell cycle scoring was applied. UMAP dimensionality reduction resulted in 15 cell subclusters, which were annotated. Monocle2 (v2.30.1) and CytoTRACE (v0.3.3) were used for pseudotime trajectory analysis, and differential gene expression was calculated with FindAllMarkers and FindMarkers. Functional enrichment analysis was based on GO and KEGG databases, and cell-cell interaction was analyzed with CellphoneDB (v2.1.1). pySENIC (v0.12.1) was used for transcription factor analysis.

2.10 Statistical analysis

All data are presented as mean $\pm$ standard deviation (x $\pm$ S). One-way ANOVA was used for comparisons among multiple groups, with P<0.05 considered statistically significant.

3. RESULTS

3.1 CTS alleviates pathological progression in a mouse model of KD

To evaluate the therapeutic effect of CTS on KD-associated vasculitis, we developed a model of LCWE-induced KD vasculitis in mice (**Figure 1A**), which effectively simulated the vascular inflammation symptoms observed in human KD. As previously reported [23], mice injected with LCWE exhibited fever symptoms than PBS-treated (normal) control mice (**Figure 1B**). Furthermore, the LCWE mice developed vascular inflammation and mild myocarditis (**Figure 1D**). Specifically, local inflammatory cell infiltration was observed around the aortic root (A_0), as indicated by arrows, along with inflammatory infiltration around the coronary arteries. Disruption of the myocardial cell structure was also noted, indicating myocardial damage. Notably, mice treated with low or high doses of CTS showed improvements in myocardial inflammation and vasculitis, along with decreased fever. Additionally, LCWE-injected mice exhibited abdominal aortic dilation, which was normalized in the CTS-treated groups (**Figure 1C**).

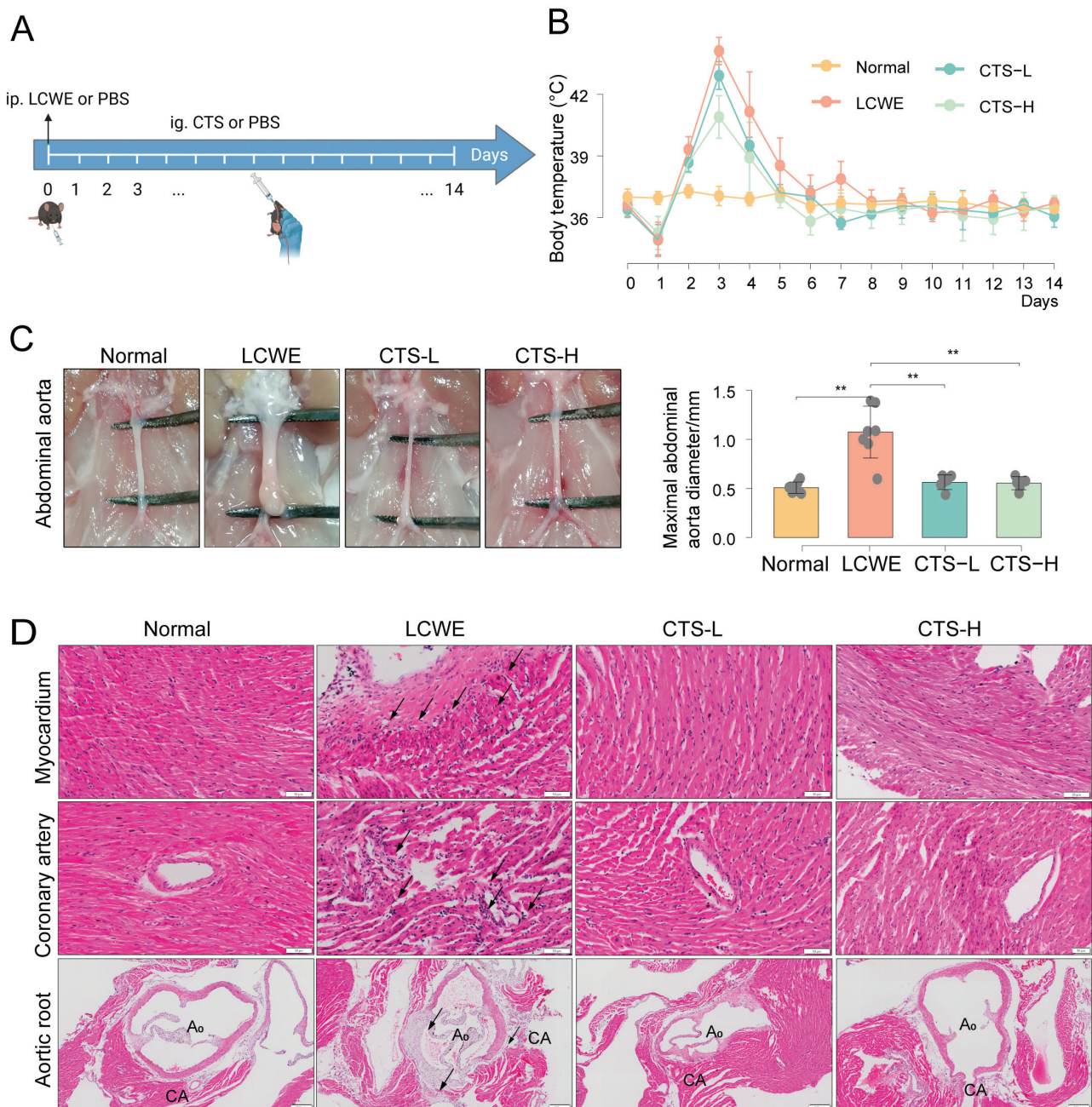


Figure 1 | CTS alleviates disease severity in the LCWE mouse KD model.

A. Schematic diagram of KD model construction in LCWE-induced KD mice. B. Body temperatures of mice in the normal, LCWE, high-dose CTS, and low-dose CTS groups during 2 weeks of model construction. C. Representative images of the abdominal area in the four groups of mice, and statistical analysis of abdominal aortic diameter. Scale bars, 200 mm. D. Representative H&E images of heart tissue, coronary arteries, and aortic root regions in the four groups of mice. Scale bars, 50 μ m.

3.2 Network pharmacology analysis indicates that the anti-KD mechanism of CTS involves modulation of cellular inflammatory chemotactic signaling pathways

To comprehensively investigate the mechanism of CTS in KD, we used network pharmacology analysis

to predict the potential interaction network of CTS in the context of KD. Initially, we gathered 126 predicted CTS targets from the TCMSP (<http://tcmspnw.com/>) and SwissTargetPrediction (<http://swisstargetprediction.ch/>) databases, then constructed a target network (Figure 2A). Concurrently, we collected 2,361 predicted

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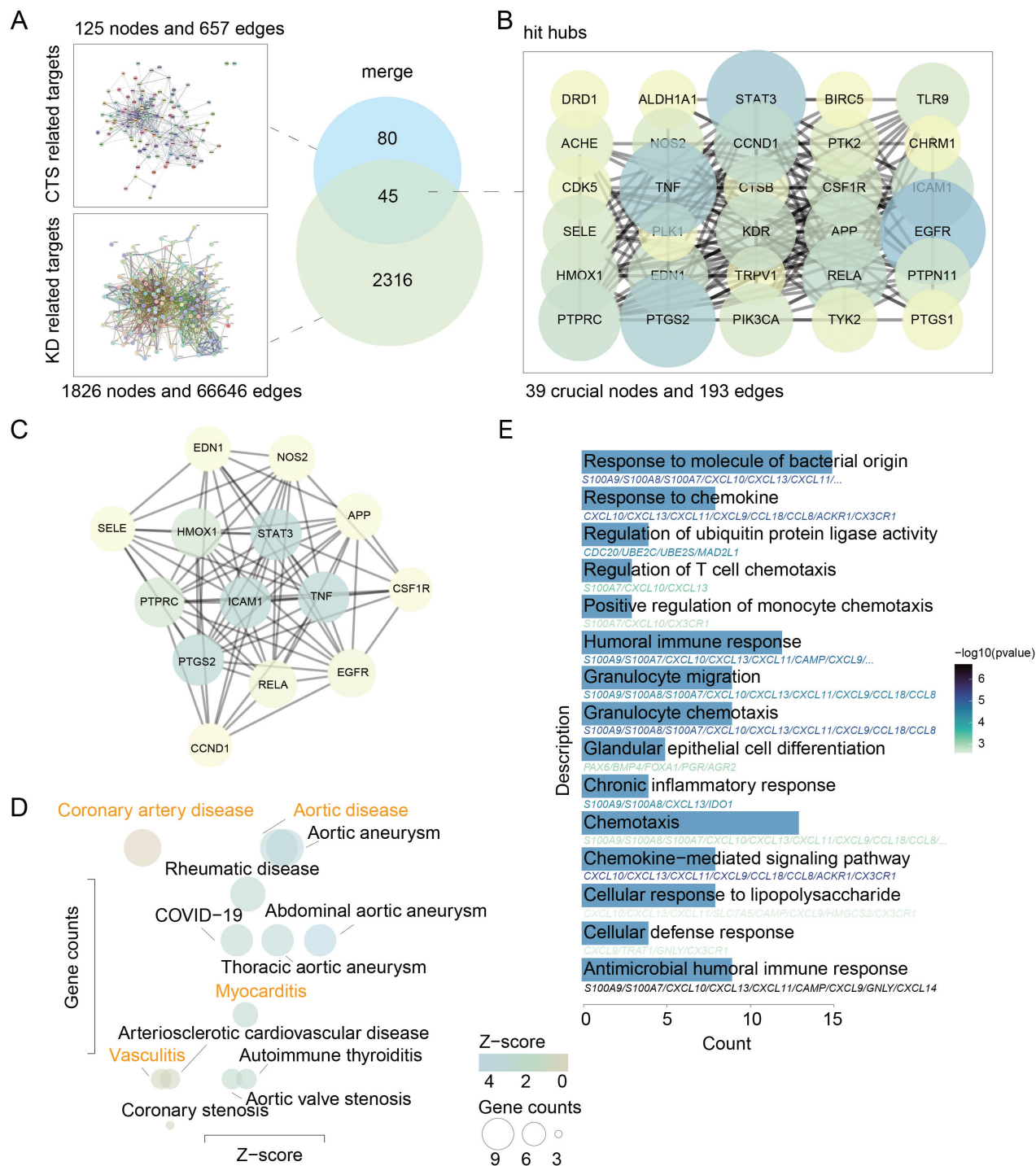


Figure 2 | Network pharmacology construction of the CTS-KD common target network.

A. A total of 126 CTS target genes and 2,361 KD target genes were obtained from public databases; after merging of the two target networks, 45 common target genes were identified. B. A CTS-KD common target network was constructed, and Cytoscape was used to calculate degree values for each target, identifying 39 key targets with degree values >4. C. The core network nodes of the CTS-KD target network were extracted, thus yielding 11 cardiovascular disease-associated genes. D. DO analysis of CTS-KD common target genes showed enrichment in various cardiovascular disease-associated conditions. E. GO analysis of CTS-KD common target genes revealed enrichment in pathways regulating immune response and chemotactic activity.

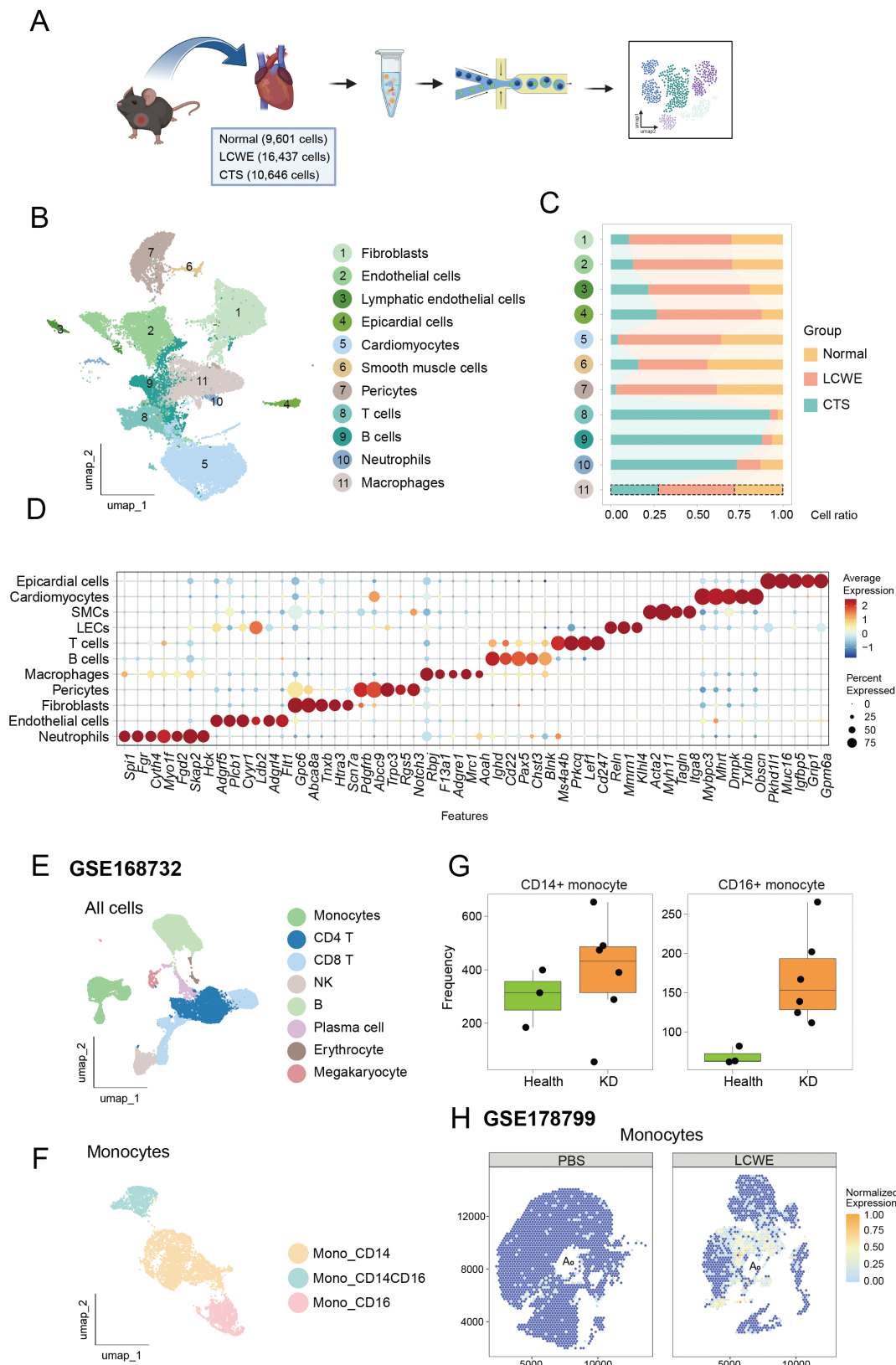


Figure 3 | CTS decreases macrophages in the LCWE model, according to snRNA-seq.

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A. Workflow diagram of snRNA-seq and data analysis for mouse heart tissue. B. Uniform Manifold Approximation and Projection (UMAP) plot displaying cell mapping for all cells from the normal, LCWE, and CTS groups, consisting of 11 cell clusters. C. Bar plot showing the proportions of each cell cluster across the normal, LCWE, and CTS groups. D. Dot plot showing representative marker genes across cell clusters. E. UMAP plot displaying the integrated cell map from GSE168732 scRNA-seq data, including peripheral blood mononuclear cells from three healthy individuals and six patients with KD. F, G. Monocytes extracted from GSE168732 were subdivided into three subtypes (CD14⁺, CD16⁺, and CD14⁺CD16⁺), with counts of CD14⁺ and CD16⁺ monocytes provided for each patient. H. Analysis of GSE178799 spatial transcriptomics data from the LCWE mouse model heart, identifying monocytes and displaying their spatial distribution in a dot plot.

KD disease targets from the OMIM (<https://omim.org/>), Digenet (<https://www.disgenet.org/>), and GeneCards (<https://www.genecards.org/>) databases, thus resulting in the creation of a similar target network (Figure 2A). From these two networks, we extracted 45 overlapping targets and constructed a shared target network for CTS and KD. By selecting nodes with a degree value more than twice the average degree of all ordinary nodes, we identified key nodes and constructed a key node network for CTS-KD (Figure 2B). This key node network highlighted 11 significant nodes marked in blue (Figure 2C), including PTGS2, which has been identified as a potential early biomarker for KD screening [24], as well as ICAM, TNF, and HOMX1, which have been associated with vascular endothelial injury and inflammation in KD [25, 26].

To further investigate the potential pathways of CTS in treating KD, we conducted DO, KEGG (Figure S1A), and GO enrichment analyses on the 45 overlapping genes within the CTS-KD target network. The DO analysis revealed enrichment in cardiovascular diseases similar to KD, including coronary artery disease, aortic disease, myocarditis, and vasculitis (Figure 2D), thus suggesting the potential of CTS to serve as a therapeutic agent for KD. Additionally, the GO analysis highlighted multiple biological processes associated with the chemotaxis of immune cells (Figure 2E). In KD, the immune system is abnormally activated, and innate and adaptive immune responses contribute to disease progression. Elevated numbers of monocytes and megakaryocytes have been observed in the peripheral blood of patients with KD [27], along with elevated levels of chemokines in the serum [28] and substantial infiltration of inflammatory cells in the aortic root of the heart [29]. RNA-seq data (GSE235994) from heart tissues of mice treated with LCWE (KD model) or PBS also indicated greater expression of chemokine-related genes in the LCWE mice (Figure S1B). Therefore, CTS might mitigate KD by disrupting the migration of inflammatory cells from the peripheral blood to the damaged vascular regions of the heart, thereby slowing pathological progression.

3.3 snRNA-seq reveals the cardiac cellular landscape in a mouse model of KD

To gain deeper insights into the mechanism of CTS in treating KD, we used snRNA-seq to analyze the heart tissues of mice in the normal, LCWE, and high-dose

CTS groups (Figure 3A). After multiple quality-control steps, we obtained sequencing data comprising 9,601, 16,437, and 10,646 cells in each group for downstream analysis, respectively. The data from the three groups were integrated, filtered for doublets, analyzed for principal components, subjected to dimensionality reduction, clustered, and annotated, thus resulting in 11 distinct cell subclusters (Figure 3B, D), which included cardiomyocytes, fibroblasts, endothelial cells, smooth muscle cells, and immune cells. Notably, we observed a greater proportion of macrophages in the heart tissues of mice in the LCWE group than the normal group. In contrast, CTS treatment decreased this proportion to levels comparable to those in the LCWE group (Figure 3C).

Pro-inflammatory macrophages in KD might originate from monocytes recruited from peripheral blood to sites of vascular inflammation or from resident cardiac macrophages. To investigate this possibility, we collected scRNA-seq data (GSE168732) from PBMCs of patients with acute KD before IVIG therapy and healthy controls (Figure 3E). The study, including three healthy individuals and six patients with KD [30], revealed a higher proportion of monocytes in the patients with KD (Figure 3F, G). In addition, we collected spatial transcriptomic data (GSE178799) from the cardiac tissues of LCWE-induced KD mouse models to examine the distribution of infiltrating monocytes [23]. The spatial transcriptomic data of mouse cardiac tissues demonstrated that cardiac macrophages localized predominantly to the aortic root (Figure 3H). In addition, LCWE stimulation upregulated the expression of inflammatory cytokines in THP-1 cells, whereas treatment with CTS resulted in a corresponding downregulation of these inflammatory cytokines (Figure S2A, B). These findings provide valuable insights into the distribution and dynamics of macrophages in KD, and suggest that CTS treatment might influence macrophage infiltration and contribute to the modulation of cardiac inflammation.

3.4 CTS decreases the transcriptional activity of MITF/TFEC in macrophages in the heart in LCWE mice and decreases chemokine transcription

We further investigated the role of macrophages in KD progression, as well as CTS's mechanism of action on these immune cells. Initially, we conducted GO enrichment analysis of macrophages from the three experimental

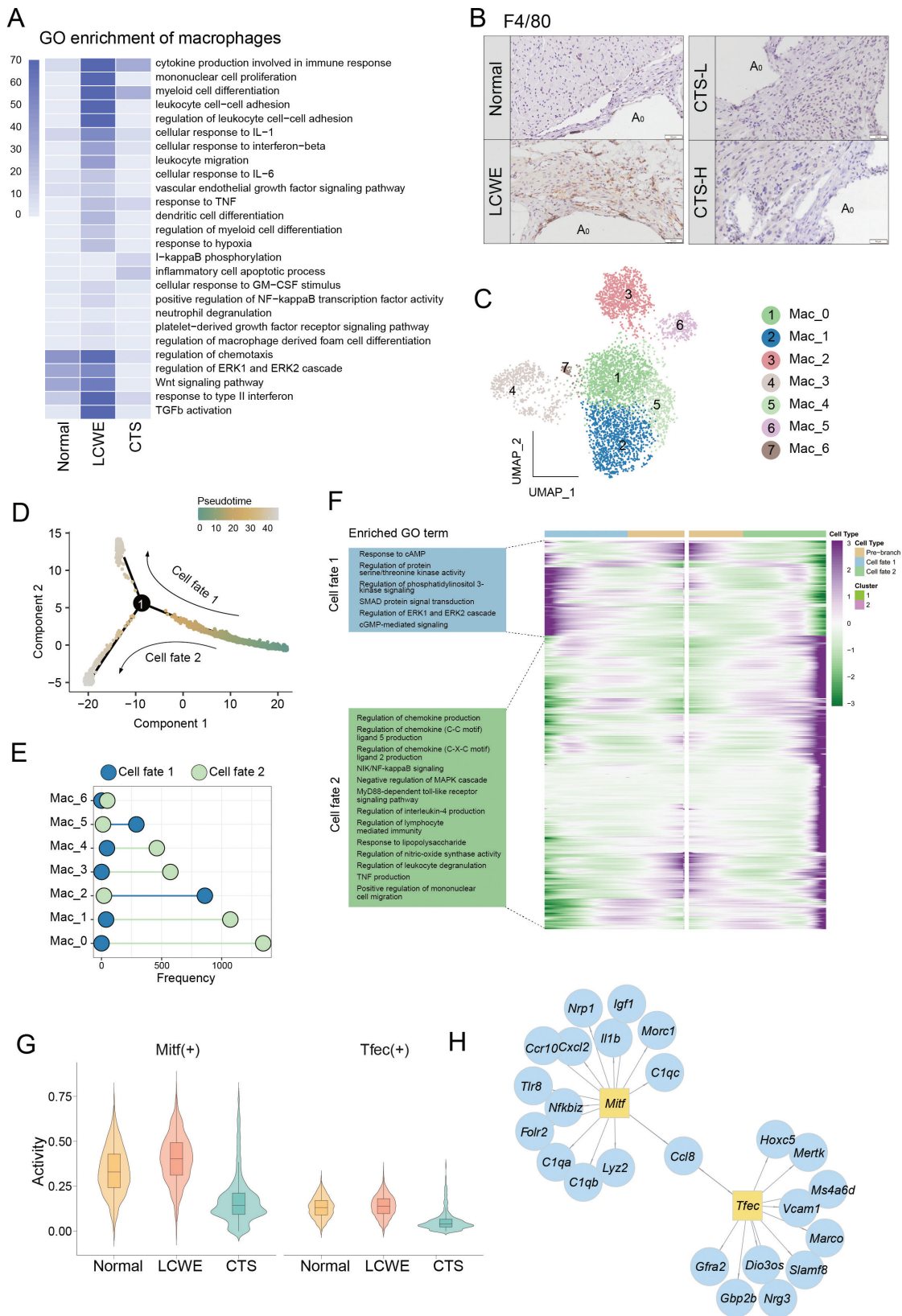


Figure 4 | Chemotactic signaling pathway enhancement in cardiac macrophages of LCWE mice is attenuated by CTS.

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A. GO analysis of all macrophages between groups in the mouse snRNA-seq data. B. Immunohistochemical staining of macrophages (F4/80 antibody) in heart tissue sections from normal, LCWE, low-dose CTS, and high-dose CTS mice. C. UMAP showing the cell map of all macrophages in the mouse snRNA-seq data. D. Pseudotime analysis combining monocytes from GSE168732 data with macrophages from mouse snRNA-seq data; human peripheral blood monocytes are positioned at the pseudotime origin, whereas mouse cardiac macrophages are distributed across two developmental branches. E. Distribution of mouse cardiac macrophages along the two trajectory branches. F. GO analysis of differentially expressed genes in the two pseudotime branches. G. Box plot showing transcription factor activity. pySENIC analysis revealed significantly enhanced activity of transcription factors *Mitf* and *Tfec* in cardiac macrophages of LCWE mice, which decreased after CTS treatment. H. Transcriptional regulatory network showing genes regulated by the transcription factors *Mitf* and *Tfec*, both of which control *Ccl8* expression.

groups. Significant enrichment was observed in pathways associated with leukocyte adhesion, cytokine production, inflammatory signaling, chemotaxis regulation, and myeloid cell differentiation in the LCWE group versus the normal and CTS-treated groups (Figure 4A). Additionally, immunohistochemical staining of the macrophage marker F4/80 in mouse heart tissues confirmed the accumulation of macrophages around the aortic root in LCWE-treated mice. In contrast, less accumulation was observed in the groups treated with low- and high-dose CTS (Figure 4B). Further clustering analysis of macrophages identified seven distinct subpopulations (Figure 4C).

Because monocytes are the first immune cells that arrive at the heart during the early stages of KD [31], we hypothesized that circulating monocytes might migrate from the peripheral blood to the heart, then differentiate into macrophages. This process might contribute to the subsequent increase in inflammation. Next, we integrated the monocyte data (GSE168732) obtained from patient PBMCs with the macrophage data derived from our mouse snRNA-seq. The monocyte data from patient PBMC scRNA-seq served as a foundation for pseudotime trajectory analysis (Figure 4D). This analysis revealed two distinct mature developmental states of mouse macrophages. One developmental branch of macrophages, designated as cell fate 2, exhibited GO functional enrichment associated primarily with chemotactic regulation (Figure 4E, F). Moreover, in the pseudotime trajectory analysis of the transition from peripheral monocytes to macrophages, we observed gradual upregulation of inflammatory and chemotactic genes associated with KD progression (Figure S3A, B). These results suggested active regulation of inflammatory and chemotactic gene expression in mature macrophages, thus potentially amplifying the inflammatory response in KD.

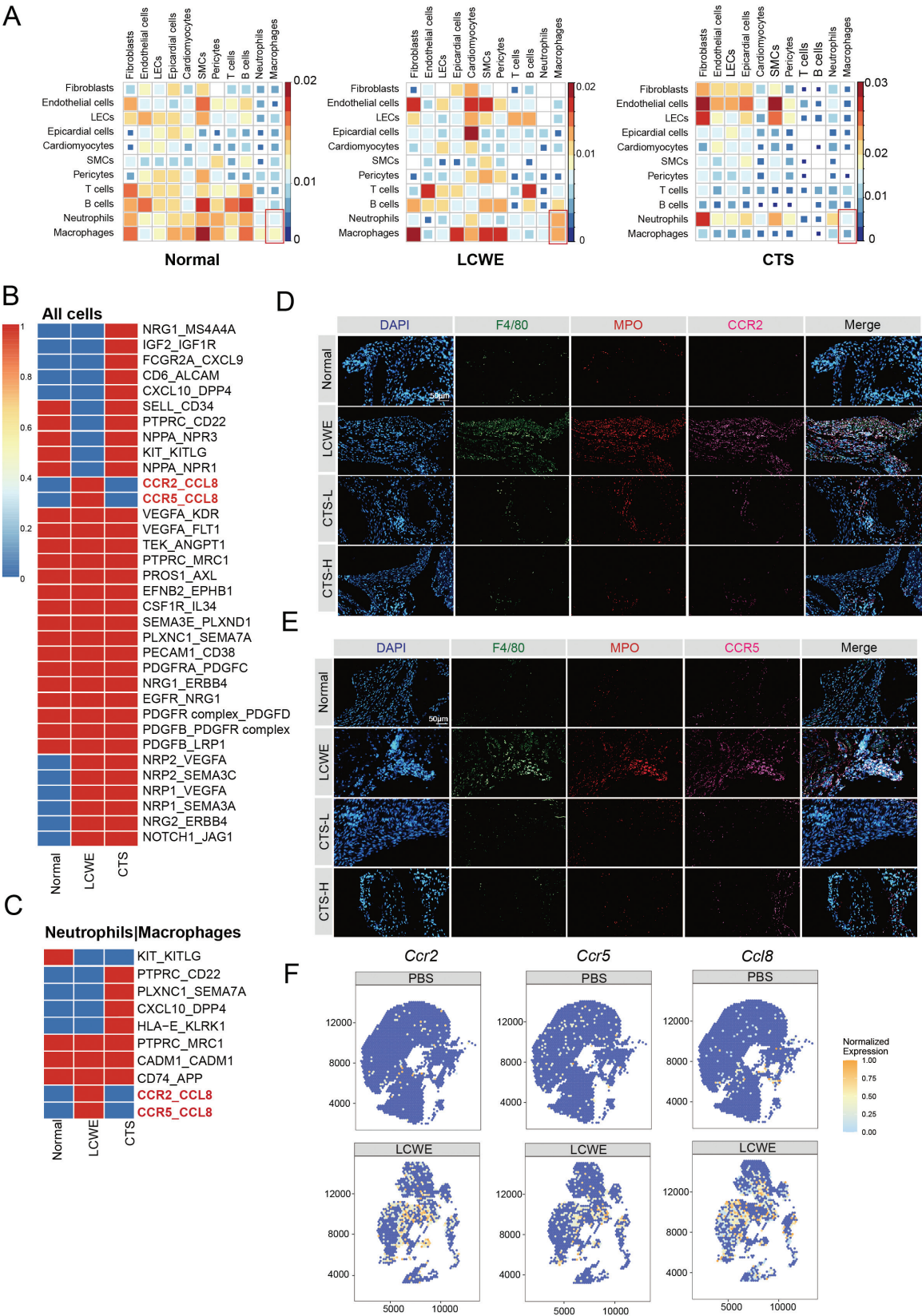
To further investigate the regulatory mechanisms underlying the enhanced inflammation and chemotactic activity of macrophages, we performed a transcriptional regulatory analysis in these cells. This analysis revealed that the cardiac macrophages of LCWE-treated mice exhibited significantly greater activity of the *Mitf* and *Tfec* transcription factors, which are part of the MiT-TFE family, than observed in the normal and CTS-treated groups (Figures 4G, S4A). The MiT-TFE family comprises basic helix-loop-helix leucine-zipper transcription factors that regulate cell differentiation,

metabolism, and stress responses [32-35]. Moreover, in paraffin sections of mouse heart tissue, we observed more pronounced colocalization of MITF and TFEC fluorescence in the LCWE group (Figure S4B). Through transcriptional regulatory network analysis (Figure 4H), we discovered that *Tfec*'s downstream targets include *Ccl8*, a crucial component in chemokine production, as well as other chemokines and inflammatory factors (Figure S4C). Treatment with the MITF transcriptional activity inhibitor TT012 led to downregulated expression of inflammatory cytokines and chemokines in THP-1 cells (Figure S4D). These findings suggested that CTS might inhibit *Ccl8* transcription in macrophages within the LCWE-induced disease environment, thereby preventing the recruitment of inflammatory cells and limiting vasculitis progression.

3.5 CTS treatment decreases the interaction intensity between macrophages and neutrophils in the LCWE model

We hypothesized that CTS might limit the recruitment of additional inflammatory cells and prevent the spread of inflammation by inhibiting the secretion of chemokines from macrophages in the heart. To investigate this possibility, we analyzed cell interactions with snRNA-seq data from mouse hearts. The heatmaps depicting cell interaction correlations across the three groups indicated a significantly stronger interaction between macrophages and neutrophils in the LCWE group than the normal and CTS-treated groups (Figure 5A). Furthermore, the analysis of ligand-receptor interactions revealed that the CCR2-CCL8 and CCR5-CCL8 receptor-ligand pairs were significantly enhanced in the LCWE group compared with the normal and CTS groups, particularly in the interactions between macrophages and neutrophils (Figure 5B, C).

Immunofluorescence staining of heart tissue sections for macrophage markers (F4/80) and neutrophil markers (MPO) revealed that both macrophages and neutrophils were present in greater numbers and showed greater spatial proximity to each other in the LCWE group than the normal and CTS-treated groups (Figure 5D, E). Additionally, CCR2 and CCR5 colocalized at sites where neutrophils and macrophages were in proximity to each other (Figure 5D, E). Spatial transcriptomics data from mouse hearts further corroborated these findings (Figure 5F). Collectively, these results suggested that CTS



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A. Heatmap showing interaction correlations among all cell groups in mouse heart snRNA-seq data. B. Heatmap displaying interaction statistics of ligand-receptor pairs among all cells in mouse heart snRNA-seq data. C. Heatmap showing interaction statistics of ligand-receptor pairs specifically between macrophages and neutrophils in mouse heart snRNA-seq data. D, E. Immunofluorescence staining of mouse heart tissue sections. Nuclei: DAPI (blue); macrophages: F4/80 (green); neutrophils: MPO (red), CCR2 or CCR5 (magenta). F. Dot plot showing spatial expression distribution and levels of the *Ccr2*, *Ccr5*, and *Ccl8* genes in the spatial transcriptomics data from the GSE178799 LCWE mouse model heart.

might attenuate the progression of vasculitis by inhibiting the recruitment of neutrophils by macrophages.

3.6 CTS attenuates the inflammatory activation of neutrophils recruited by macrophages in the heart in LCWE model mice

To further investigate the roles of neutrophils recruited by macrophages in vasculitis, we analyzed neutrophil data from mouse hearts. Neutrophils were categorized into five subpopulations (Figure 6A). The Neu_1 subpopulation exhibited high expression of *Ccr2* and *Ccr5* (Figure 6B). Neu_1 was situated in the later stages of the pseudotime differentiation trajectory of all neutrophils (Figures 6D–F, S5A) and was the most active group of inflammatory neutrophils (Figures 6C, S5B). We investigated the effects of CTS treatment on neutrophil chemotaxis and inflammation, and identified differentially expressed genes between the CTS and LCWE groups. Notably, *Palld* expression was downregulated in the CTS group (Figure 6G, H). *Palld* plays a crucial role in neutrophil functions, including migration, adhesion, and chemokine secretion [36], and might be involved in the CTS-mediated inhibition of neutrophil chemotaxis. On the basis of these findings, we concluded that CTS effectively decreases the population of inflammation-active neutrophils in the mouse heart, thereby halting the progression of vasculitis.

4. DISCUSSION

KD affects primarily the coronary and aortic arteries in children, thereby leading to abnormal activation and imbalances within the immune system [10, 37–40]. Activation of the innate immune response is a major driver of KD-related vasculitis and is characterized by an increase in innate immune cells in peripheral blood during the acute phase of the disease [41]. Neutrophil infiltration subsequently occurs and progressively advances from the intima to the adventitia of the coronary arteries, thus resulting in necrotizing arteritis [9]. Typically, during inflammatory stimulation, circulating monocytes migrate to the affected tissue, where they differentiate into macrophages that participate in inflammation regulation and tissue repair [42–44]. In the CAWS-induced KD mouse model, monocytes migrate to the heart on the first day of disease induction [31], alongside activated tissue-resident macrophages [9], which promote the recruitment of neutrophils and monocytes, thereby exacerbating cardiac inflammation. However, the factors initiating the abnormal activation of the innate

immune system and the migration of peripheral monocytes toward the cardiac vasculature remain unclear. Miyabe et al. have reported that aortic-resident macrophages use Dectin-2 to recognize antigenic signals and release CCL2, thereby recruiting monocytes [45].

In our LCWE mouse snRNA-seq data, we observed the activation of macrophages in cardiac tissue. Notably, our pseudotime trajectory analysis traced the progression of circulating monocytes to cardiac macrophages, and revealed gradual upregulation of inflammatory and chemotactic genes, including CCL8. This transition suggested that mature macrophages play critical roles in amplifying inflammation in KD. These findings align with prior studies demonstrating the importance of monocyte-derived macrophages in the pathogenesis of cardiovascular inflammation [46, 47]. To further explore the regulatory mechanisms underlying this transition, we performed transcriptional regulatory analysis, which highlighted the MiT-TFE family, particularly MITF and TFEC, as key regulators in LCWE-induced macrophages. These transcription factors drive the expression of genes such as *Ccl8*, and consequently contribute to the recruitment of inflammatory cells and exacerbation of the inflammatory response. The transcriptional activity of these macrophages was altered, thus promoting the recruitment of inflammatory neutrophils and worsening cardiac vasculitis.

We demonstrated that CTS effectively inhibited the recruitment of *Ccr2*⁺*Ccr5*⁺ neutrophils by macrophages, thereby suppressing vasculitis. CTS intervention in the LCWE-induced KD mouse model ameliorated myocarditis, coronary arteritis, and inflammation surrounding the aortic root. Analysis of snRNA-seq data from the hearts of LCWE-injected mice treated with CTS revealed a diminished proportion of macrophages. Previous studies have demonstrated that CTS mitigates inflammation by modifying macrophage metabolic reprogramming [48]. In our study, CTS treatment altered the activity of macrophage transcription factors and notably decreased the activity of *Tfec* and *Mitf*, and subsequently chemokine secretion and the recruitment of inflammatory neutrophils. Therefore, CTS not only influences the phenotypic transition of macrophages but also affects their interactions with other inflammatory cells, thereby attenuating inflammation progression.

Although our study highlights the role of macrophage transcriptional regulation in KD-related inflammation and the therapeutic potential of CTS, further experimental validation is needed to confirm the molecular mechanisms underlying these findings. Specifically, additional

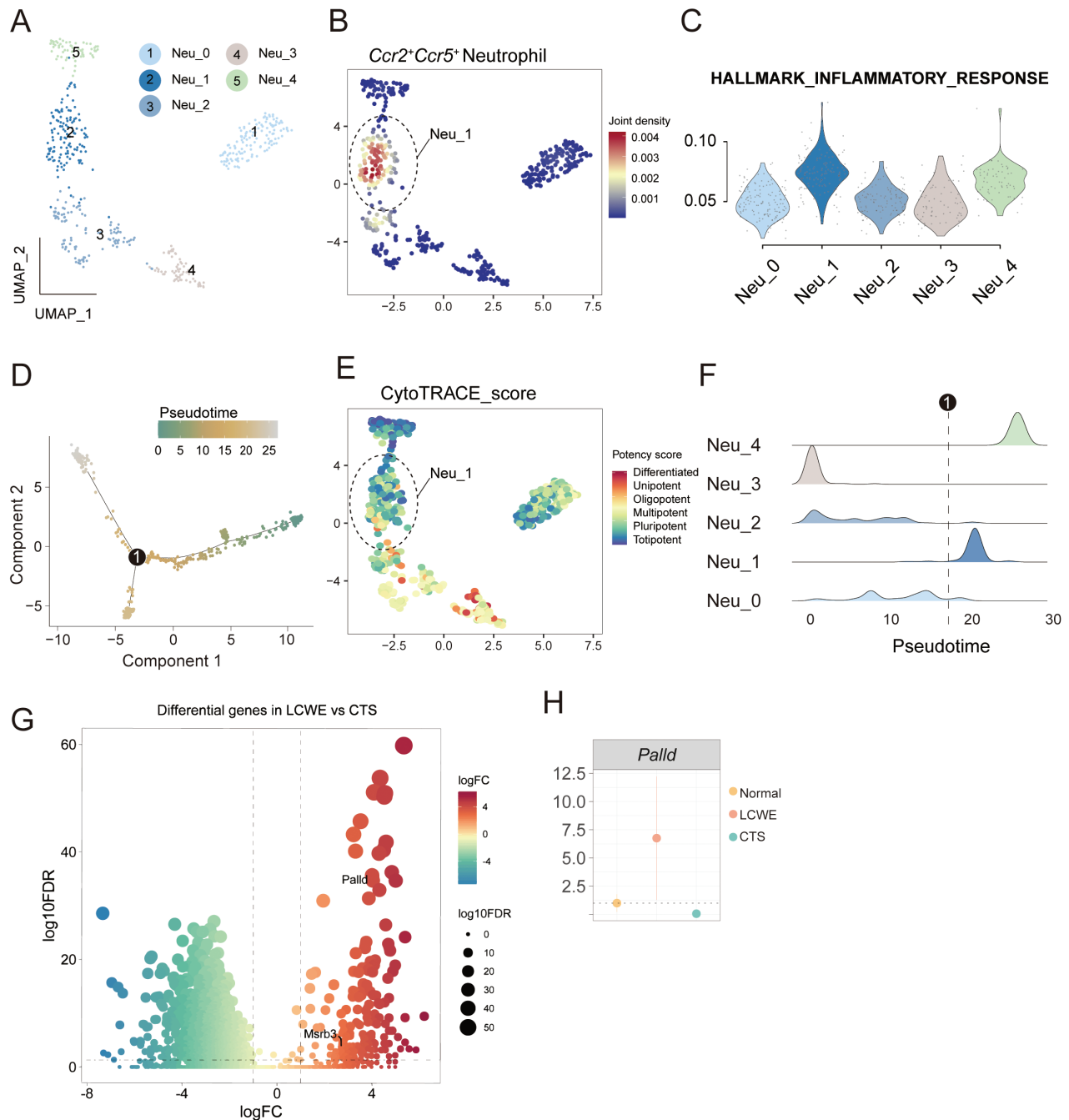


Figure 6 | CTS attenuates inflammatory activation of neutrophils in the heart in LCWE mice.

A. UMAP showing the cell map of neutrophils in mouse heart snRNA-seq data. B. Dot plot showing the distribution of a subset of neutrophils (Neu_1) with high *Ccr2* and *Ccr5* expression. C. Inflammatory response scores of each neutrophil subset in the mouse heart. D. Pseudotime analysis of neutrophils in the mouse heart. E. Differentiation status of neutrophils in the mouse heart, calculated with cytoTRACE, showing a gradual decline in stemness along the totipotent-pluripotent-multipotent-oligopotent-unipotent-differentiated trajectory. F. Ridge plot showing the distribution of neutrophil subpopulations along pseudotime in the mouse heart. G. Volcano plot illustrating the differentially expressed genes between LCWE and CTS treatments in neutrophils derived from mouse heart snRNA-seq data. H. Expression levels of *Palld* under various conditions (normal, LCWE, and CTS) in neutrophils from the mouse heart.

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studies are required to dissect the direct effects of CTS on transcriptional regulators such as MITF and TFEC, and their downstream targets, such as Ccl8, in macrophages.

5. CONCLUSIONS

Our findings demonstrate that CTS exerts anti-inflammatory effects in KD by modulating macrophage transcriptional activity and disrupting the recruitment of inflammatory cells. Our results provide new insights into the regulation of macrophage-mediated inflammation and highlight the potential of targeting transcriptional regulators, such as MITF and TFEC, as therapeutic strategies for KD.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal experimental protocols complied with the ethical guidelines of the Ethics Committee of Nanjing Medical University Affiliated Children's Hospital (ethics approval number 202105054-1).

DATA AVAILABILITY

The snRNA-seq data are available for reproducing the results. All R scripts used to process data are available from the corresponding author on request.

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SUPPLEMENTARY MATERIAL

Supplementary Material can be downloaded from https://amm-journal.org/wp-content/uploads/2025/03/amm20240076_Suppl.pdf.

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