

Hypothermic machine perfusion protects DCD graft liver from ischemia-reperfusion injury by enhancing macrophage efferocytosis via KLF2-NLRP3 signaling

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Abstract. Donation after circulatory death (DCD) is a key source of liver grafts but it is associated with more severe ischemia-reperfusion injury (IRI) and poorer transplant outcomes compared with donation after brain death. Hypothermic machine perfusion (HMP) effectively decreases DCD graft injury, but its protective molecular mechanisms remain unclear. Kruppel-like factor 2 (KLF2) is an endothelial protective transcription factor induced by hemodynamic mechanical stimulation. However, the role of KLF2 in IRI during HMP in DCD livers is unclear. Rat livers undergoing DCD modeling followed by static cold storage (CS) or HMP were used to assess KLF2 expression and macrophage efferocytosis. Injury was assessed by serum alanine transferase/aspartate transferase levels, histology, TUNEL apoptosis assay and immunofluorescence (IF) for *in situ* efferocytosis. Protein markers were analyzed via western blotting, immunohistochemistry and IF. *In vitro*, HUVECs and macrophages were subjected to simulated CS/reperfusion. Macrophages efferocytosis was quantified using fluorescently labeled apoptotic Jurkat cells. Mechanisms were explored by RNA sequencing and co-immunoprecipitation. Compared with the CS group, HMP decreased pathological

injury, apoptosis and inflammation in DCD liver injury. KLF2 expression was upregulated. However, knockdown of KLF2 abrogated these endothelial protective effects *in vitro*. Furthermore, overexpression of KLF2 enhanced macrophage efferocytosis, whereas suppression of KLF2 impaired this. Moreover, enhanced efferocytosis contributed to inflammation resolution, ultimately improving overall graft injury and decreasing apoptosis. Mechanistically, KLF2 inhibited the NOD-like receptor protein 3 (NLRP3) inflammasome to suppress pyroptosis, thereby indirectly enhancing efferocytosis. HMP alleviated IRI in DCD liver grafts by upregulating endothelial KLF2, which inhibited NLRP3 inflammasome-mediated pyroptosis, thereby improving the inflammatory microenvironment and promoting macrophage efferocytosis.

Introduction

Liver transplantation is the treatment of choice for end-stage liver disease (1). However, the rising prevalence of liver disease has led to a severe shortage of donor organs, which limits the broader clinical application of this procedure (2). The estimated global age-standardized liver disease-associated mortality rates increased from 1990 to 2021 across 112 countries, rising from 103.4 deaths per 1,000,000 people in 1990 to 173.0 deaths per 1,000,000 people in 2021 (3). Donation after circulatory death (DCD) has expanded the donor pool by using marginal grafts, which refer to organs with higher transplant risks and borderline quality due to factors such as donor age, pathological conditions, or prolonged ischemia time (4). However, DCD liver grafts are vulnerable to more severe ischemia-reperfusion injury (IRI). IRI in liver transplantation refers to tissue damage that occurs during the ischemic period and is exacerbated following restoration of blood flow. This triggers an inflammatory response, impairs hepatic microcirculation and induces hepatocyte injury, thereby hindering the functional recovery and long-term survival of the transplanted liver (5,6).

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To mitigate IRI, several dynamic graft preservation techniques have been developed, with hypothermic machine perfusion (HMP) serving as one of the most promising strategies (7). Compared with static cold storage (CS), HMP better protects donor organs, decreases the severity of IRI and promotes graft repair, thereby helping to alleviate the organ shortage crisis (8). However, the protective mechanism of HMP against IRI in DCD liver grafts has not yet been fully elucidated.

Kruppel-like factor 2 (KLF2) is a transcription factor that maintains endothelial cell (EC) quiescence. Its expression in ECs is upregulated in response to physiological laminar shear stress (LSS) from blood flow, activating a transcriptional program that confers vascular protection (9). Conversely, under CS conditions, where hemodynamic stimulation is absent, the endothelial KLF2-mediated protective pathway is significantly downregulated, leading to loss of the vascular protective phenotype (10,11). Previous studies have demonstrated that during organ preservation, HMP maintains a form of physiological laminar flow, which upregulates KLF2 expression in ECs and exerts protective effects (12,13). Our preliminary study has also confirmed that pharmacologically induced overexpression of KLF2 in donors can alleviate IRI in rat DCD livers (14). Yet, the specific role of KLF2 during HMP in the context of DCD liver IRI requires further elucidation.

Macrophage efferocytosis refers to the process by which macrophages efficiently clear apoptotic cells, thereby preventing a larger inflammatory cascade and maintaining immune balance and organismal stability (15). This process also plays a key role in modulating hepatic IRI (16,17). Macrophages can adapt their phenotype and function in response to signals from the microenvironment (18,19). Such microenvironmental cues typically originate from intercellular communication. For example, ECs shape macrophage phenotypes through the secretion of paracrine factors (20,21). Exosomes secreted by vascular ECs under physiologically protective LSS polarizes macrophages toward an anti-inflammatory phenotype (22). Moreover, exosomes derived from KLF2-upregulated ECs modulate macrophage behavior regulate macrophages by enhancing immune-modulatory responses and attenuating pro-inflammatory responses (23). Additionally, in atherosclerosis, disturbed blood flow leads to the downregulation of KLF2 expression (24,25), which is associated with impaired macrophage efferocytosis (26-28). Simvastatin and resveratrol enhance macrophage efferocytosis (29,30). It was hypothesized that upregulation of EC KLF2 expression during HMP may alter the microenvironment via paracrine signaling, thereby enhancing macrophage efferocytosis. This mechanism may represent a novel pathway contributing to the protective effects of HMP on DCD liver grafts.

Materials and methods

Animals and treatments. A total of 48 adult male Sprague-Dawley (SD) rats aged 8-10 weeks (body weight, 250-300 g) were purchased from Hubei Research Center of Laboratory Animals. They were housed under room temperature of 20-25°C, humidity of 50-70% and a 12/12-h light-dark cycle. They were fed standard chow and provided with adequate water freely and housed in a specific pathogen-free

facility. All animal procedures were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Research Council (31) and approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Nanchang University (Nanchang, China; approval nos. CDYFY-IACUC-202211QR219 and CDYFY-IACUC-202407QR199).

As previously described (5), a DCD rat model was established. Briefly, rats were anesthetized by intraperitoneal injection of 30 mg/kg sodium pentobarbital. Without portal vein clamping or prior heparinization, the diaphragm was transected to induce cardiac arrest. Rats were placed on a thermostatic heating pad to maintain liver temperature at 29±1.45°C. After 30 min, *in situ* perfusion of the liver was performed at 4°C using 50 ml histidine-tryptophan-ketoglutarate (HTK) solution through the abdominal aorta to flush out the blood. The portal vein and suprahepatic inferior vena cava were cannulated, while the infrahepatic inferior vena cava and right adrenal vein were ligated. Finally, the liver was excised.

Rats were randomly divided into groups (n=6/group; Fig. 1): Sham, cardiac arrest was not induced, but all other procedures were performed as aforementioned; CS, following DCD modeling, the isolated liver was preserved in a 4°C University of Wisconsin (UW) solution for 24 h; HMP, liver was preserved in a 4°C UW solution for 23 h, followed by 1 h HMP and 1 h normothermic perfusion (NMP); CS + putrescine (PU), rats were provided with drinking water supplemented with 3 mM PU (32) (cat. no. HY-Y1781; MedChemExpress); and HMP + UNC2025, SD rats received a single intraperitoneal injection of the c-mer tyrosine kinase (MerTK) inhibitor UNC2025 (cat. no. HY-12344; MedChemExpress) at a dose of 10 mg/kg 1 h prior to DCD induction. Subsequently, the livers were processed, preserved, and perfused as aforementioned. The HMP and isolated perfused rat liver system have been described previously (33). Portal vein perfusion pressure was 4 mmHg, the flow rate was controlled at 0.1 ml/g·min, and the HMP duration was 1 h. Under NMP, the perfusate temperature was maintained at 37±1°C, the portal vein perfusion pressure was ≤8 mmHg, the perfusion flow rate was controlled at ~1 ml/g·min, and the reperfusion duration was 1 h (33,34). HMP was conducted using UW solution, while reperfusion was performed with HTK solution. At the end of NMP, perfusate and liver tissue samples were collected for further analysis. The water for both the control and PU-supplemented groups was changed every 2-3 days. After 2 weeks, venous blood was collected via inferior vena cava puncture under anesthesia (administered via intraperitoneal injection of 30 mg/kg pentobarbital sodium), with ~1 ml drawn per animal for liver function analysis, followed by liver retrieval as aforementioned.

Euthanasia was performed by an overdose of sodium pentobarbital (>100 mg/kg, administered intraperitoneally) to ensure a painless procedure and compliance with animal ethics guidelines. The rats were checked for cardiac arrest, respiratory cessation, body stiffness and pupillary dilation to confirm death.

Liver injury assessment. The collected serum and perfusate following normothermic reperfusion were stored at -80°C. Alanine transferase (ALT) and aspartate transferase (AST)

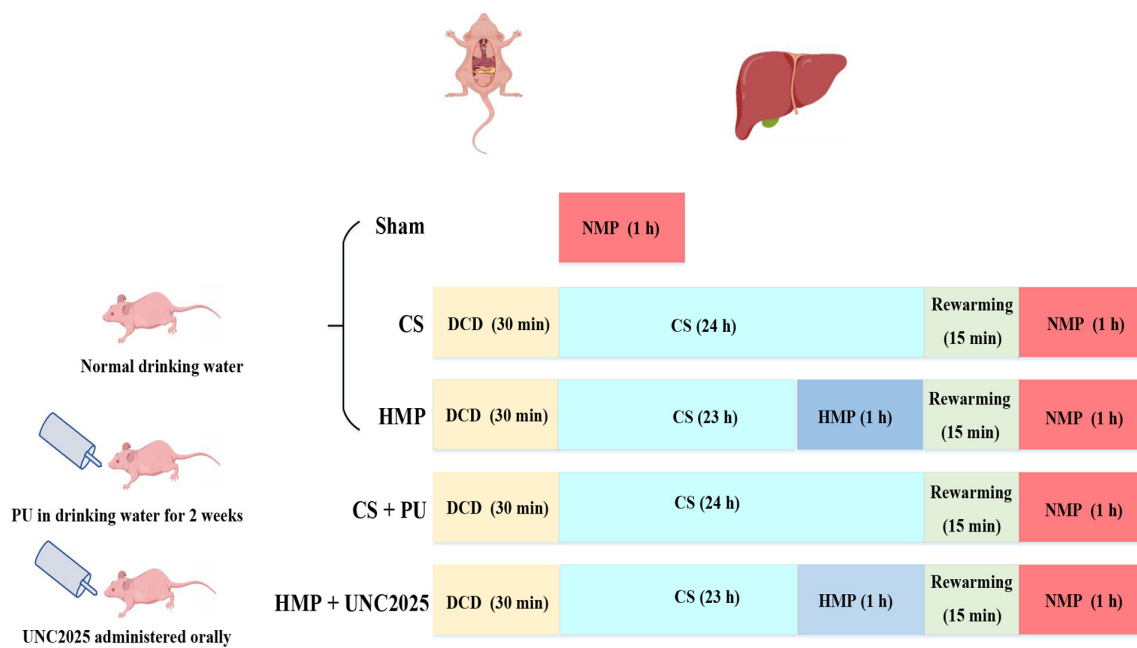


Figure 1. Experimental design and timeline. The timeline of key procedural steps is illustrated, including the initiation of the DCD process, organ procurement, preservation period and reperfusion phase. NMP, normothermic machine perfusion; CS, cold storage; HMP, hypothermic machine perfusion; DCD, donation after circulatory death; PU, putrescine.

concentration in rat serum and perfusate was assessed using the BS-2000 automatic biochemical analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd.). The liver tissues were fixed using 4% buffered paraformaldehyde at room temperature for 24 h. The paraffin-embedded tissues were sectioned at a thickness of 4 μ m. Staining was performed with hematoxylin for 5 min and eosin for 15 min, both at room temperature. Finally, morphological observation and image acquisition were conducted under a light microscope and graded using the Suzuki scoring system (35) in a blinded manner. Suzuki scoring system was assessed on a scale from 0 to 4 based on the severity of cellular vacuolization, sinusoidal congestion and hepatocyte necrosis.

TUNEL assay and *in situ* efferocytosis measurement. Following fixation in 4% paraformaldehyde (24 h, room temperature), the paraffin-embedded liver sections were stained for hepatocyte apoptosis using the terminal deoxynucleotidyl TUNEL assay kit (cat. no. G1504; Wuhan Servicebio Technology Co., Ltd.). Immunofluorescence (IF) staining was performed using the TUNEL assay kit and anti-CD68 antibody (1:1,000; cat. no. ab303565, Abcam). The sections were incubated with TUNEL staining reagent at room temperature for 60 min, followed by three washes with PBS. Sections were blocked with 10% normal goat serum at room temperature for 30 min (Beijing Solarbio Science & Technology Co., Ltd.) and incubated overnight at 4°C with the anti-CD68 antibody. Finally, the slides were incubated with a fluorescently labeled secondary antibody (1:1,000; cat. no. ab205718; Abcam) at 37°C for 1 h and counterstained with DAPI before applying antifade mounting medium (cat. no. 0100-01, SouthernBiotech). Fluorescence microscopy (Olympus Corporation) was used

to observe the slides and TUNEL⁺ nuclei associated with CD68⁺ macrophages were counted to quantify *in situ* efferocytosis. Number of macrophages per field of view: 30-50. All researchers responsible were blinded to the group allocation.

Immunohistochemistry (IHC) and IF staining. Following fixation in 4% paraformaldehyde (24 h, room temperature), rat liver tissues were embedded in paraffin and cut into 4 μ m-thick sections. Paraffin-embedded specimens were sectioned, deparaffinized with xylene and rehydrated in decreasing concentrations of ethanol. Add proteinase K working solution, then incubate the slides at 37°C for 20 min for antigen retrieval. Endogenous enzymes were blocked by incubating with 3% H₂O₂ at room temperature for 20 min. The slides were blocked with 10% normal goat serum at room temperature for 1 h for IHC staining. The slides were incubated overnight at 4°C with the primary antibody. The HRP-conjugated secondary antibody Goat Anti-Rabbit IgG H&L (1:1,000; cat. no. ab205718, Abcam) was applied for 1 h at room temperature. The primary antibodies were as follows: Anti-caspase 3 (1:200; cat. no. 9661, Cell Signaling Technology, Inc.), anti-myeloperoxidase (MPO; 1:1,000; cat. no. ab208670, Abcam), anti-KLF2 (1:200; cat. no. bs-2772R, BIOSS) and anti-T cell immunoglobulin and mucin domain-containing protein 4 (TIMD4; 1:200; cat. no. a17807, ABclonal Biotech Co., Ltd.). The DAB working solution was then applied to the tissue sections for chromogenic development. Cell nuclei were counterstained with Mayer's hematoxylin (cat. no. G1080; Beijing Solarbio Science & Technology Co., Ltd.) for 1 min at room temperature, and the slides were observed under a microscope. The staining results were quantitatively analyzed using the image analysis software Image J (6.0, National Institutes of Health).

Transmission electron microscopy (TEM). Fresh liver tissue and cell samples (size, 1–2 mm²) were fixed in 2.5% glutaraldehyde (Wuhan Servicebio Technology Co., Ltd.) at 4°C for 24 h. Cell samples were pelleted by centrifugation at 2,000 × g (4°C, 5 min) in an EP tube. The supernatant and glutaraldehyde fixative were removed. A total of ~1 mm³ of melted 1% agarose was added to the pellet and gently mixed. After cooling and solidification, the resulting agarose-embedded cell block was processed for subsequent steps. The samples were dehydrated through a gradient of ethanol and acetone and embedded (37°C, overnight) in epoxy resin. Samples were sectioned into thin slices (60–80 nm), which were placed on copper grids. Finally, the sections were performed with 2% uranyl acetate (room temperature) for 8 min, followed by 2% lead citrate (both room temperature) for 8 min. The images were analyzed using TEM (cat. no. JEM1400, JEOL Ltd.). All researchers were blinded to the group allocation information.

Cell culture and treatment. Human umbilical vein ECs (PUMC-HUVEC-T1, cat. no. CL-0675), THP-1 (human monocytic leukemia cell line; cat. no. CL-0233) and Jurkat (cat. no. CL-0129; all Procell Life Science & Technology Co., Ltd.) cells were cultured at 37°C in a 5% CO₂ humidified incubator. HUVECs were cultured in DMEM (cat. no. G4511; Wuhan Servicebio Technology Co., Ltd.) with high glucose, supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (cat. no. A5256701; Gibco; Thermo Fisher Scientific, Inc.). THP-1 and Jurkat cells were cultured in THP-1 cell culture medium (cat. no. CM-0233) and Jurkat cell culture medium (cat. no. CM-0129; both Procell Life Science & Technology Co., Ltd.), respectively. THP-1 cells were cultured (37°C) in medium supplemented with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; cat. no. HY-18739, MedChemExpress) for 48 h to induce differentiation into macrophages. To establish the CS/reperfusion (CS/Rep) model, the cell culture medium was replaced with UW solution and the cells were incubated at 4°C for 24 h. Subsequently, the solution was replaced with high-glucose DMEM (cat. no. G4511; Wuhan Servicebio Technology Co., Ltd.) containing FBS (cat. no. A5256701; Gibco; Thermo Fisher Scientific, Inc.; 10%), and the cells were incubated at 37°C with 5% CO₂ for 3 h to simulate the reperfusion process. Nigericin was dissolved in DMSO and diluted in DMEM containing 10% FBS. To verify that the pyroptosis of ECs affects macrophage phagocytosis, HUVECs were treated (37°C, 24 h) with nigericin (cat. no. HY-100381, MedChemExpress; 0, 5, 10 and 20 μM) to induce pyroptosis.

In vitro shear stress experiment construction. HUVECs (1 × 10⁶) were seeded on collagen-coated glass slides to form a monolayer. As previously described (36), the slides were placed into a flow chamber system, and DMEM supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) was injected into the chamber to cover the ECs. The flow rate was regulated using a unidirectional flow pump and ECs on the slides were exposed to LSS at 12 dyne/cm² for 0, 3, 6 and 12 h before performing cell viability assay.

Lentivirus construction. The recombinant lentivirus for KLF2 overexpression (ov-KLF2) was designed and constructed by

OBiO Technology (Shanghai) Corp., Ltd. KLF2 knockdown was achieved using lentiviral particles expressing short hairpin RNA targeting KLF2 (sh-KLF2) with vector backbone (cat. no. GV493; both Shanghai GeneChem Co., Ltd.), and the concentration of nucleic acid used was 20 μg. The sequence of sh-KLF2 was 5'-GCCTTCGGTCTCTTCGACGAC; the sequence-3' of sh-control is 5'-TTCTCCGAACGTGTCACGT-3'. To transduce HUVECs, the lentivirus was added to wells containing cells in serum-free and antibiotic-free DMEM along with the transfection reagent Lipofectamine® (cat. no. 11668019; Thermo Fisher Scientific). The mixture was incubated at 37°C for 48 h. Stable cell lines were obtained following puromycin (cat. no. BL528A, Biosharp Life Sciences) selection for subsequent experiments. The incubation period between transfection and subsequent treatment was 72 h. To verify whether NLRP3 inhibition rescues the phenotypes resulting from KLF2 deficiency, sh-KLF2-transfected cells were treated (37°C) with 10 μM NLRP3 inhibitor MCC950 (cat. no. HY12815, MedChemExpress) for 24 h and subjected to CS/Rep as aforementioned. Expression levels of pyroptosis-associated proteins were assessed by western blotting.

Cell viability assay. Treated HUVECs were washed three times with PBS, then incubated (37°C with 5% CO₂) with calcein-AM/propidium iodide (PI; cat. no. L6037, Suzhou Youyilandi Biotechnology Co., Ltd.) for 30 min, according to the manufacturer's instructions. Following fixation with 4% formaldehyde at room temperature for 15 min, the cells were observed under a fluorescence microscope.

Flow cytometry. 1 × 10⁵ HUVECs were collected in flow tubes and centrifuged at 1,000 × g at 4°C for 5 min. The supernatant was discarded, and the cells were resuspended in pre-cooled PBS. Following an additional centrifugation step as aforementioned, the cell pellet was resuspended in 1X binding buffer (Wuhan Servicebio Technology Co., Ltd.). The Annexin V-FITC/PI Apoptosis Detection kit (Wuhan Servicebio Technology Co., Ltd.) was used to measure early and late apoptosis of HUVECs. Briefly, cells were stained with Annexin V-FITC/PI at 37°C for 30 min. HUVECs were washed twice with PBS and the stained cells were resuspended in cold PBS. Analysis was performed using a flow cytometer (CytoFlex S, Beckman Coulter, Inc.) and data were analyzed using FlowJo software (v10.8; BD Biosciences).

Indirect co-culture. In a Transwell co-culture system, 0.2 ml HUVECs at a concentration of 1 × 10⁴ cells/ml was added to the upper chamber, while 0.5 ml PMA-induced macrophages (THP-1 derived) at a concentration of 1 × 10⁴ cells/ml was seeded in the lower chamber. High-glucose DMEM supplemented with 10% FBS was used as the culture medium for both monoculture and co-culture conditions. All groups were subjected to indirect co-culture. HUVECs in the upper chamber were removed, while macrophages in the lower chamber were retained for phagocytosis assay.

Induction of apoptosis in Jurkat cells and in vitro phagocytosis assay. To induce apoptosis in Jurkat cells, the cells were exposed to UV light (254 nm) for 15 min, followed by incubation under normal cell culture conditions for

Table I. Primer sequences of the target genes.

Gene	Primer name	Primer sequence, 5'→3'
IL-1 β (rat)	Forward	AATCTCACAGCAGCATCTCGACAAG
	Reverse	TCCACGGGCAAGACATAGGTAGC
IL-6 (rat)	Forward	ACTTCCAGCCAGTTGCCTTCTTG
	Reverse	TGGTCTGTTGTGGGTGGTATCCTC
TNF- α (rat)	Forward	AAAGGACACCATGAGCACGGAAAG
	Reverse	CGCCACGAGCAGGAATGAGAAG
KLF2 (human)	Forward	GGCTGCGGCTGGAAGTTTG
	Reverse	GCACAGATGGCACTGGAATGG
NLRP3 (human)	Forward	GCTTGCCGACGATGCCTTCC
	Reverse	GCTGTCAATTGTCCTGGTGTCTTCC
β -actin (human)	Forward	CAGATGTGGATCAGCAAGCAGGAG
	Reverse	CGCAACTAAGTCATAGTCCGCCTAG
β -actin (rat)	Forward	GCTGTGCTATGTTGCCCTAGACTTC
	Reverse	GGAACCGCTCATTGCCGATAGTG

KLF2, Kruppel-like factor 2.

2-3 h to obtain apoptotic Jurkat cells (ACs) (37). ACs were labeled with 5-chloromethylfluorescein diacetate (CMFDA) (cat. no. 40721ES) and macrophages were labeled with CellTracker™ Red CMTPX dye (CMTPX) (cat. no. 40717ES; both Shanghai Yeasen Biotechnology Co., Ltd.), according to the manufacturer's instructions. Labeled ACs were added to the macrophages at a ratio of 5:1, followed by co-incubation (37°C, 5% CO₂) for 45 min. Cells were washed three times with PBS to remove unbound ACs. The macrophages were fixed (37°C) with 4% formaldehyde for 20 min, followed by three washes with PBS. The ability of macrophages to phagocytose ACs was visualized using IF microscopy. For microscopic analysis, ≥ 3 fields of view per sample were examined, and ≥ 30 macrophages/field were evaluated. The phagocytic index was calculated using the following formula: (Number of macrophages containing apoptotic cells/total number of macrophages) $\times 100\%$. Data were normalized to the control group (set to 100%).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from liver tissue and HUVECs using TRIzol (cat. no. 15596026CN; Thermo Fisher Scientific, Inc.). RT was performed using Hifair®V one-step RT-gDNA digestion SuperMix kit (cat. no. 11142ES10; Shanghai Yeasen Biotechnology co., Ltd.). as follows: Primer annealing at 30°C for 5 min, cDNA synthesis 55°C for 30 min and the reaction was terminated by heating at 85°C for 5 min. SYBR Green (Biosharp Life Sciences) was used for qPCR. Thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec (denaturation) and 60°C for 30 sec (combined annealing/extension), with fluorescence signal measurement at the end of each extension step. β -actin was used as the reference gene. Relative expression of target genes was calculated according to the 2^{- $\Delta\Delta$ Cq} method (38). The primers for all target genes are listed in Table I.

Western blotting. The western blotting procedure was performed as previously described (6). The primary antibodies were as follows: Anti-KLF2 (1:1,000; cat. no. 23384-1-AP, Proteintech Group, Inc.), anti-KLF2 (1:1,000; cat. no. PAB40163; Bioswamp; Wuhan Bientle Biotechnology Co., Ltd.), anti-BAX (1:1,000; cat. no. 50599-2-Ig), anti-BCL-2 (1:1,000; cat. no. 60178-1-Ig; both Proteintech Group, Inc.), anti-p53 upregulated modulator of apoptosis (1:1,000; PUMA; cat. no. A3752), anti-growth arrest and DNA damage-inducible α (1:500; GADD45A; cat. no. A13487; both ABclonal Biotech Co., Ltd.), anti-NLRP3 (1:1,000; cat. no. ab283819, Abcam), anti-gasdermin D (1:1,000; GSDMD; cat. no. 20770-1-AP, Proteintech Group, Inc.), anti-caspase1 (1:1,000; cat. no. BD-PC0002; Suzhou Botron Immunotherapy Co., Ltd.), anti-IL-18 (1:1,000; cat. no. 10663-1-AP, Proteintech Group, Inc.) and anti- β -actin (cat. no. AC026, ABclonal Biotech Co., Ltd.).

Co-immunoprecipitation (Co-IP). Liver tissue was homogenized in ice-cold lysis buffer (cat. no. P2179S, Beyotime Institute of Biotechnology) supplemented with protease inhibitors. The lysates were centrifuged at 12,000 $\times$ g for 15 min at 4°C and the supernatants were collected for protein quantification using the BCA assay. Using the Protein A+G Magnetic Beads Immunoprecipitation kit (cat. no. P2179S, Beyotime Institute of Biotechnology), 200 μ l lysate was centrifuged at 10,000-14,000 $\times$ g and 4°C for 5 min. The supernatant was collected, and Protein A+G magnetic beads were magnetically separated with the supernatant removed. Subsequently, 500 μ l antibody working solution (prepared at a final antibody concentration of 30 μ g/ml) was added to resuspend the beads, followed by incubation on a rotary mixer at room temperature for 1 h. Protein A+G magnetic beads coupled with either the target antibody or normal IgG were added to the protein samples at a ratio of 20 μ l bead suspension per 500 μ l protein sample, and the mixture was incubated overnight at 4°C on a rocking platform or rotary mixer. The mixture was placed on

a magnetic stand for 10 sec to allow bead separation, and the supernatant was discarded. After incubation, the complexes were placed on a magnetic stand for 10 sec to allow bead separation. The eluted proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes for western blot analysis, as aforementioned. Then the membranes were probed with anti-NLRP3 (1:1,000; cat. no. ab283819, Abcam), or anti-KLF2 (1:1,000; cat. no. 23384-1-AP, Proteintech Group, Inc.) at 4°C overnight to detect interacting proteins. IgG was used as a negative control to confirm the specificity of the immunoprecipitation.

RNA sequencing (RNA-seq). RNA-seq was performed to elucidate the global transcriptomic alterations in response to simulated IRI across varying levels of KLF2 expression. Stable ov-KLF2 HUVECs were generated via lentiviral transduction, with empty vector-transduced cells serving as the control (ov-control). Following the CS/Rep treatment, total RNA was extracted from three biological replicates/group. RNA was extracted from samples using TRIzol (cat. no. 15596026CN; Thermo Fisher Scientific Inc.), and its integrity was assessed using the Agilent 2100 Bioanalyzer. mRNA was enriched via poly(A) selection, fragmented and reverse-transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen, cat. 1896649, USA), according to the manufacturer's instructions. A cDNA library was constructed using the Hieff NGS EvoMax RNA Library Prep kit (Shanghai Yeasen Biotechnology Co., Ltd.; cat. no. 12340ES97), with a final library concentration of 5 nM. High-throughput sequencing was performed on the Illumina platform using the NovaSeq X Series 25B Reagent kit (300 cycles; Illumina, Inc.; cat. no. 20104706) for paired-end 150 bp sequencing, with an insert size of 300–400 bp. The sequencing orientation was 3'-end sequencing. The polyA tail of mRNA was captured using Oligo(dT) magnetic beads, followed by sequencing of the fragmented sequences near the 3' end. Following quality control of the raw sequencing data, differentially expressed genes (DEGs) were identified using DESeq2 (1.22.2; github.com/mikelove/DESeq2). Then sequence quality was verified using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>, 0.11.9), including the Q20, Q30 and GC-content of the clean data. DEGs were defined as absolute log2 fold change ≥ 1 and an adjusted P-value < 0.05 . Gene Ontology (GO; geneontology.org/) analysis was performed (significance threshold: $P < 0.05$).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 8.0.1 (Dotmatics). All data are expressed as mean $\pm$ SD of ≥ 3 biological replicates. Normality was assessed using the Shapiro-Wilk test and homogeneity of variances was evaluated with Levene's test. For comparisons between two groups, unpaired Student's t-test (or Mann-Whitney U test for non-normal data) was used. For comparisons among > 2 groups, one- or two-way ANOVA followed by Tukey's post hoc test was applied. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

HMP improves DCD donor liver injury. To assess the protective effect of HMP on DCD donor livers, reperfusion fluid of

the CS and HMP groups was analyzed. Compared with the CS group, the HMP group showed a significant reduction in AST and ALT levels (Fig. 2A and B). Additionally, staining showed milder liver sinusoidal congestion, less lobular edema and reduced hepatocyte vacuolation and necrosis in the HMP group compared with the CS group (Fig. 2C and D). TEM also showed that mitochondria displayed visible cristae and less swelling and vacuolation in the HMP group compared with CS group (Fig. 2E).

To validate the protective effect of HMP on inflammation, IF staining was performed to detect neutrophil infiltration marker MPO. Neutrophil infiltration was significantly higher in the CS than in the HMP group (Fig. 3A and B). Furthermore, the mRNA expression levels of inflammatory factors such as IL-6, IL-1 β and tumor necrosis factor (TNF)- α were significantly elevated in the CS group compared with the HMP group (Fig. 3C–E).

To assess apoptosis rates in liver tissue, d TUNEL staining was performed and IHC was performed to detect caspase-3 expression. The number of TUNEL- and caspase-3-positive cells in the HMP group was significantly lower compared with the CS group (Fig. 3F–I). Furthermore, the expression of anti-apoptotic proteins (BAX/BCL2) and pro-apoptotic proteins (PUMA, GADD45A) in the HMP group, indicating a lower apoptotic level compared with the CS group (Fig. 3J–M). These findings suggest that HMP mitigated IRI in DCD donor livers.

HMP protects ECs via shear stress-induced KLF2 upregulation. To investigate whether the protective effect of HMP on DCD liver grafts was due to sustained shear stress leading to KLF2 upregulation in ECs, IF staining was performed. Liver tissue was labeled with KLF2 antibodies. The results showed that the colocalization of KLF2 fluorescence in liver sinusoidal ECs (LSECs) was significantly higher in the HMP than in the CS group (Fig. 4A and B). Western blot analysis confirmed that KLF2 protein expression was significantly higher in the HMP group compared with the CS group (Fig. 4C and D).

A parallel plate flow chamber system was used to simulate *in vivo* shear stress conditions *in vitro* (Fig. 4E) to validate that KLF2 expression is specifically induced by ECs under laminar flow conditions. Calcein-AM/PI staining was performed at 0, 3, 6 and 12 h, showing no significant differences in EC damage caused by shear stress at these time points (Fig. 4F and G). Western blot analysis of cell samples revealed that KLF2 expression increased with duration of laminar flow (Fig. 4H and I). These findings suggested that HMP may protect DCD liver grafts through upregulation of KLF2. Additionally, the protective phenotype of EC KLF2 upregulation under CS/Rep conditions was verified. *In vitro* experiments using an EC CS/Rep model revealed that overexpression of KLF2 decreased apoptosis, whereas knockdown of KLF2 increased apoptosis (Fig. 4J–L). These results suggested that the protective effect of HMP on DCD livers was closely associated with the upregulation of KLF2.

KLF2 upregulation in ECs promotes macrophage efferocytosis. To explore how the upregulation of KLF2 in ECs, induced by HMP, promotes macrophage efferocytosis, TUNEL-positive

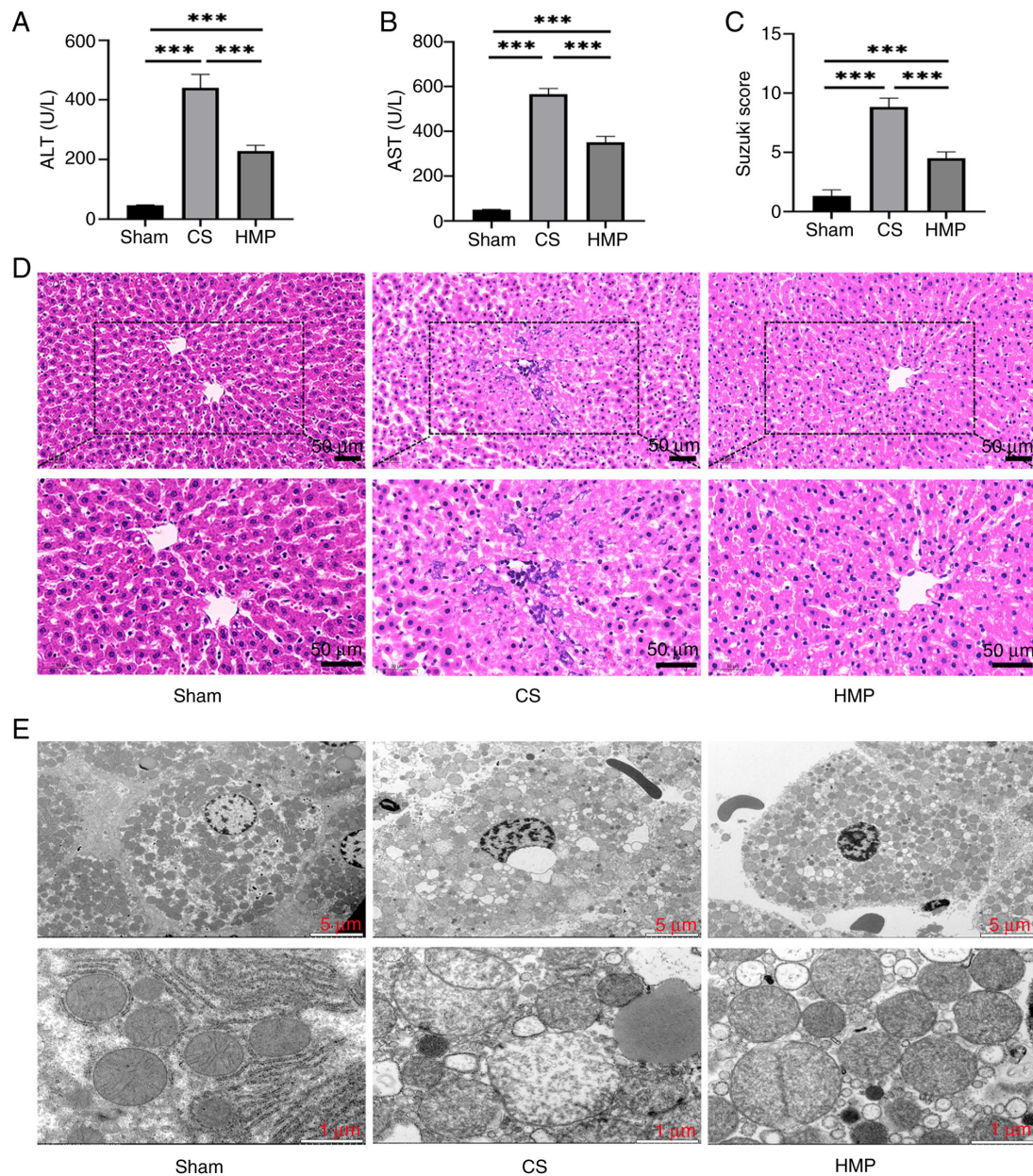


Figure 2. HMP improves donation after circulatory death donor liver injury. Levels of (A) ALT and (B) AST. (C) Histological scores were analyzed based on Suzuki's criteria (n=6). (D) Hematoxylin and eosin staining of rat liver tissue. (E) Hepatocyte microsomal modification was investigated via transmission electron microscopy (n=3). ***P<0.001. HMP, hypothermic machine perfusion; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CS, cold storage.

apoptotic cells and CD68-positive macrophages were quantified in DCD liver grafts. Fluorescence colocalization was performed to assess macrophage efferocytosis *in vivo*. Compared with the CS group, the HMP group exhibited increased efferocytosis in hepatocytes. (Fig. 5A and B).

To confirm that KLF2 upregulation in ECs promoted macrophage efferocytosis, PMA-induced THP-1-derived macrophages were co-cultured with HUVECs expressing different levels of KLF2 (Fig. 5C). Under the Transwell non-contact co-culture condition, both cell types were placed in a cold IR model to simulate their interaction during cold IR. ACs were co-incubated with macrophages. The present study assessed macrophage efferocytosis *in vitro* by quantifying the co-positive expression of CMTPX (red tracer for macrophages) and CMFDA (green tracer for ACs). Macrophages co-incubated with ov-KLF2-expressing HUVECs showed

significantly higher efferocytosis compared with the ov-control group (Fig. 5D-F). However, compared with the sh-control group, the sh-KLF2 group decreased efferocytosis. TIMD4, a marker of macrophage efferocytosis ability, expression was detected. Comparison of TIMD4 fluorescence intensity among these groups confirmed these findings (Fig. 5G-I). Specifically, macrophages in the ov-KLF2 group showed higher TIMD4 intensity than those in the ov-control group; compared with the sh-control group, the TIMD4 fluorescence intensity of macrophages in the sh-KLF2 group was significantly lower. These results indicated that the positive effect of HMP on DCD livers was mediated by upregulation of KLF2 expression to promote efferocytosis.

Increased macrophage efferocytosis decreases hepatocellular injury in DCD liver grafts. PU is an agonist that promotes

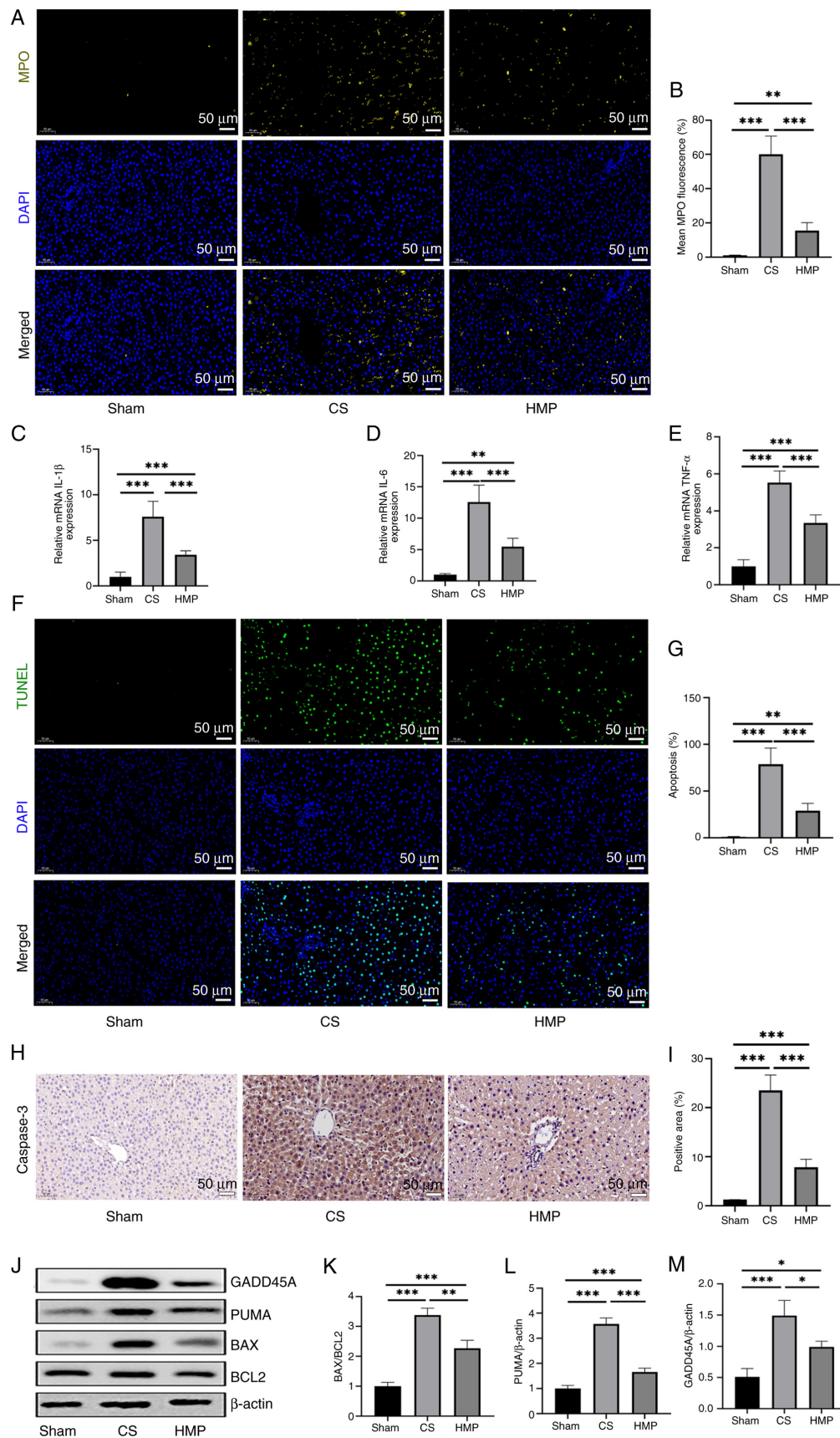


Figure 3. HMP inhibits hepatocyte apoptosis in donation after circulatory death donor livers. (A) IF staining of MPO was performed on paraffin-embedded rat liver tissues. (B) IF of MPO was quantified. Expression levels of mRNA for (C) IL-1 β , (D) IL-6 and (E) TNF- α in liver tissue were quantified using reverse transcription-quantitative PCR. (F) Hepatocyte apoptosis was assessed using TUNEL assay. (G) Number of TUNEL-positive cells was quantified. (H) Immunohistochemical staining of caspase-3 was performed on paraffin-embedded rat liver tissues. (I) Caspase-3-positive cells were quantified (n=6). (J) Expression levels of (K) BAX/BCL2, (L) PUMA and (M) GADD45A in rat liver tissue were assessed using western blotting (n=3). *P<0.05, **P<0.01, ***P<0.001. HMP, hypothermic machine perfusion; IF, immunofluorescence; MPO, myeloperoxidase; PUMA, P53 upregulated modulator of apoptosis; GADD45A, growth arrest and DNA damage-inducible alpha; CS, cold storage.

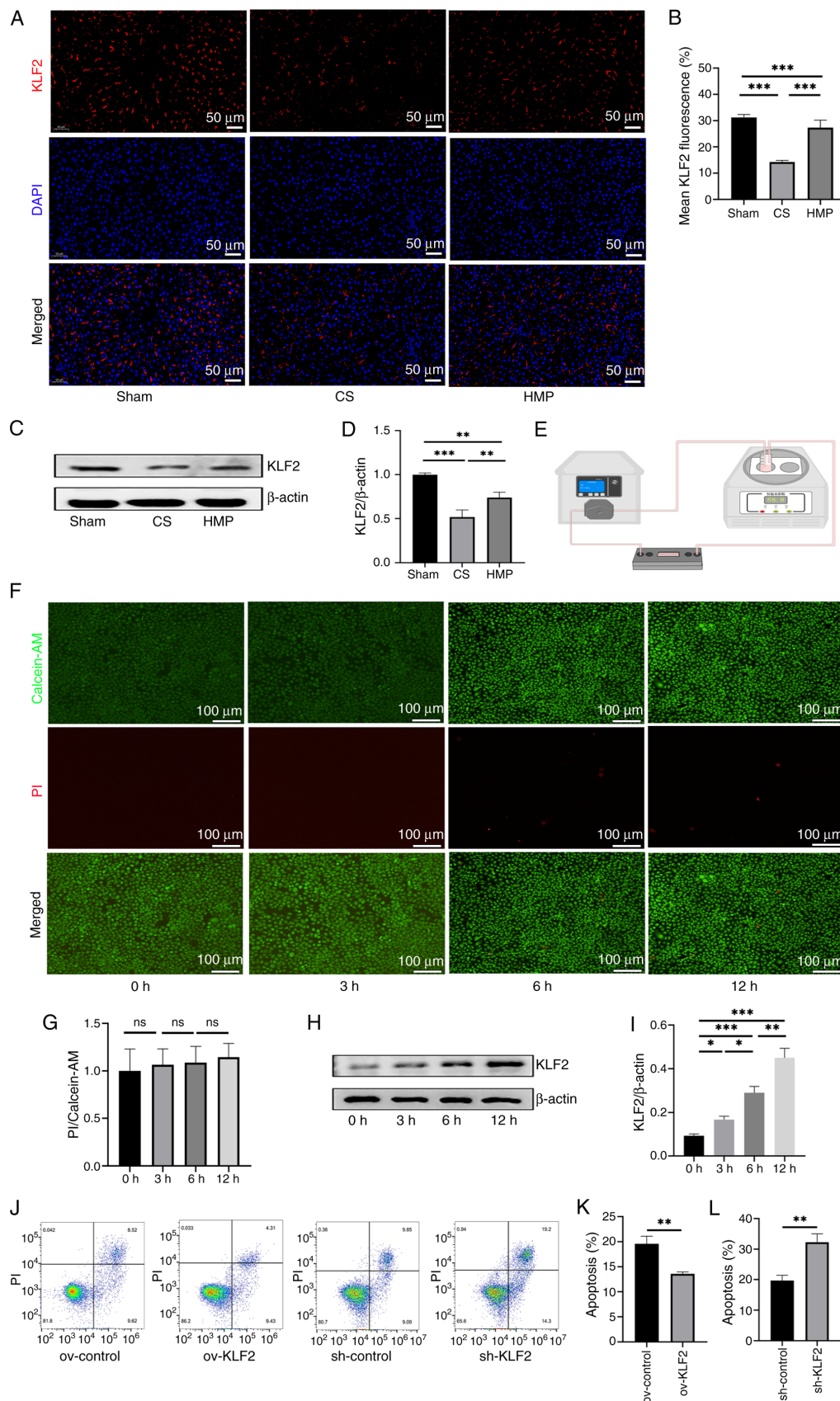


Figure 4. HMP-induced shear stress upregulates KLF2 expression. (A) Representative fluorescence staining showing the presence of KLF2 in rat liver tissue. (B) Number of KLF2-positive cells (n=6). (C) Expression levels of KLF2 in rat liver tissue were assessed using western blot analysis and (D) quantified using a Gel-Pro Analyzer. (E) Schematic diagram of an *in vitro* parallel plate flow chamber simulating *in vivo* laminar flow stimulation. (F) HUVECs were stained with Calcein-AM/PI to assess (G) cell viability. (H) Expression levels of KLF2 protein in HUVECs subjected to laminar flow stimulation *in vitro* were assessed using western blot analysis and (I) quantified using a Gel-Pro Analyzer. (J) Representative flow cytometry of apoptosis in CS/Rep model HUVECs (K) Apoptosis from HUVECs of ov-control and ov-KLF2 groups was performed using Image-pro plus 6.0. (n=3). (L) Statistical analysis of apoptosis from HUVECs of sh-control and sh-KLF2 groups. was performed using Image-pro plus 6.0 (n=3). *P<0.05, **P<0.01, ***P<0.001. HMP, hypothermic machine perfusion; KLF2, Kruppel-like Factor 2; HUVEC, human umbilical vein endothelial cell; ov, overexpression; sh, short hairpin ; CS, cold storage.

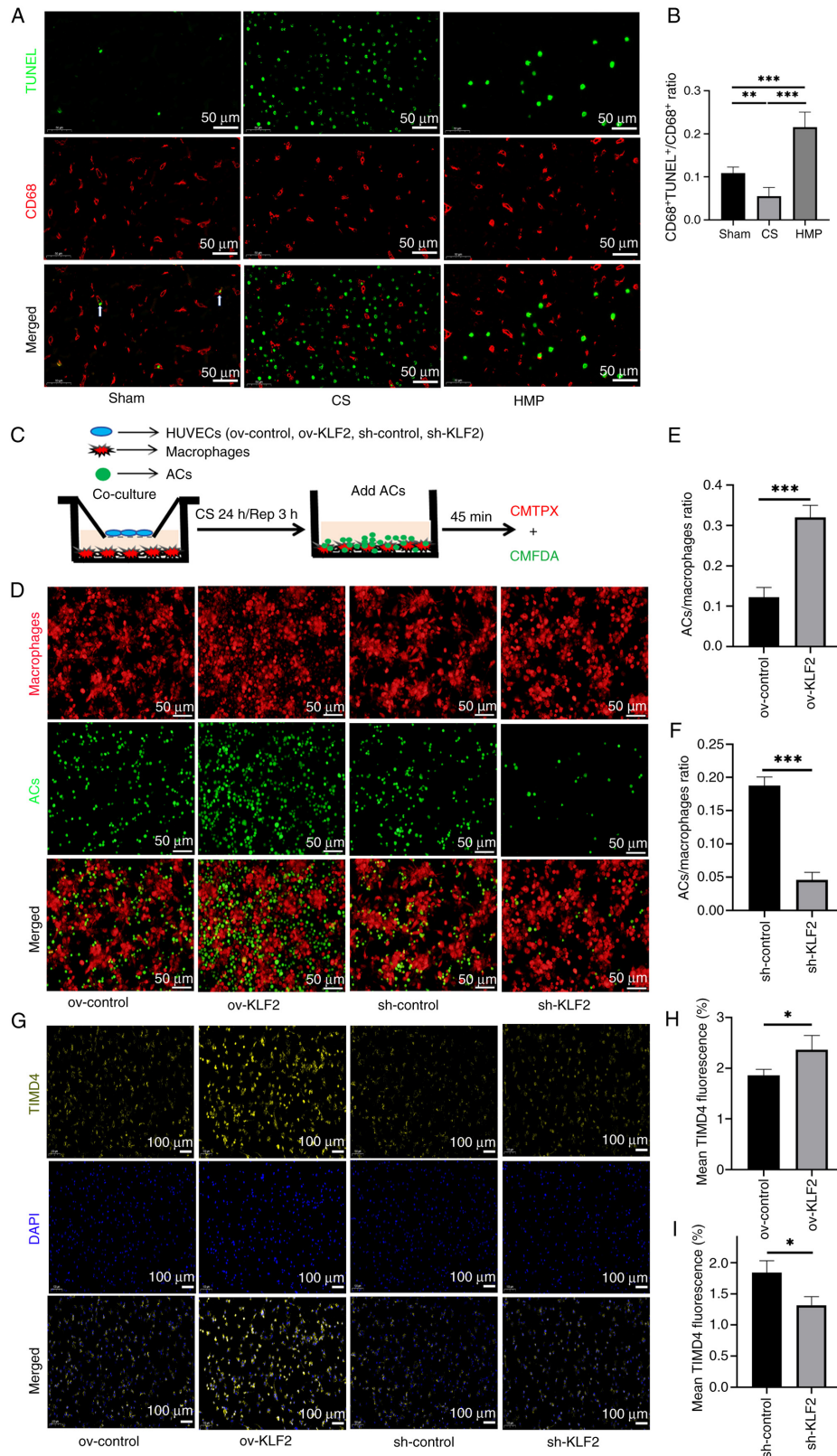


Figure 5. Upregulation of the endothelial cell protective molecule KLF2 promotes macrophage efferocytosis. (A) *In vivo* assessment and (B) quantification of efferocytosis (proportion of CD68-positive cells engulfing TUNEL-positive apoptotic cells relative to the total CD68-positive cell population). Arrows represent macrophage efferocytosis events. (C) Schematic diagram of the co-culture protocol for HUVECs with varying KLF2 expression levels and macrophages. (D) Macrophages (CMTPIX⁺) were co-cultured with apoptotic Jurkat cells (CMFDA⁺) for 45 min, and efferocytosis was quantitatively assessed *in vitro* by fluorescence co-localization analysis. (E) Statistical analysis of fluorescence signals from HUVECS of ov-control and ov-KLF2 groups was performed using Image-Pro Plus 6.0 software (n=3). (F) Statistical analysis of fluorescence signals from HUVECS of sh-control and sh-KLF2 groups was performed using Image-Pro Plus 6.0 software (n=3). (G) Representative staining of TIMD4. (H) Immunofluorescence of TIMD4 from HUVECS of ov-control and ov-KLF2 groups was quantified using Image-pro Plus 6.0. (I) Immunofluorescence of TIMD4 from HUVECS of ov-control and ov-KLF2 groups was quantified using Image-pro Plus 6.0 (n=3). *P<0.05, **P<0.01, ***P<0.001. HMP, hypothermic machine perfusion; KLF2, Kruppel-like Factor 2; AC, apoptotic Jurkat cell; HUVEC, human umbilical vein endothelial cells; CMTPIX, CellTracker™ Red CMTPIX dye; CMFDA, 5-Chloromethylfluorescein Diacetate; CS, cold storage; Rep, reperfusion; TIMD4, T-cell immunoglobulin and mucin domain-containing protein 4; ov, overexpression; sh, short hairpin.

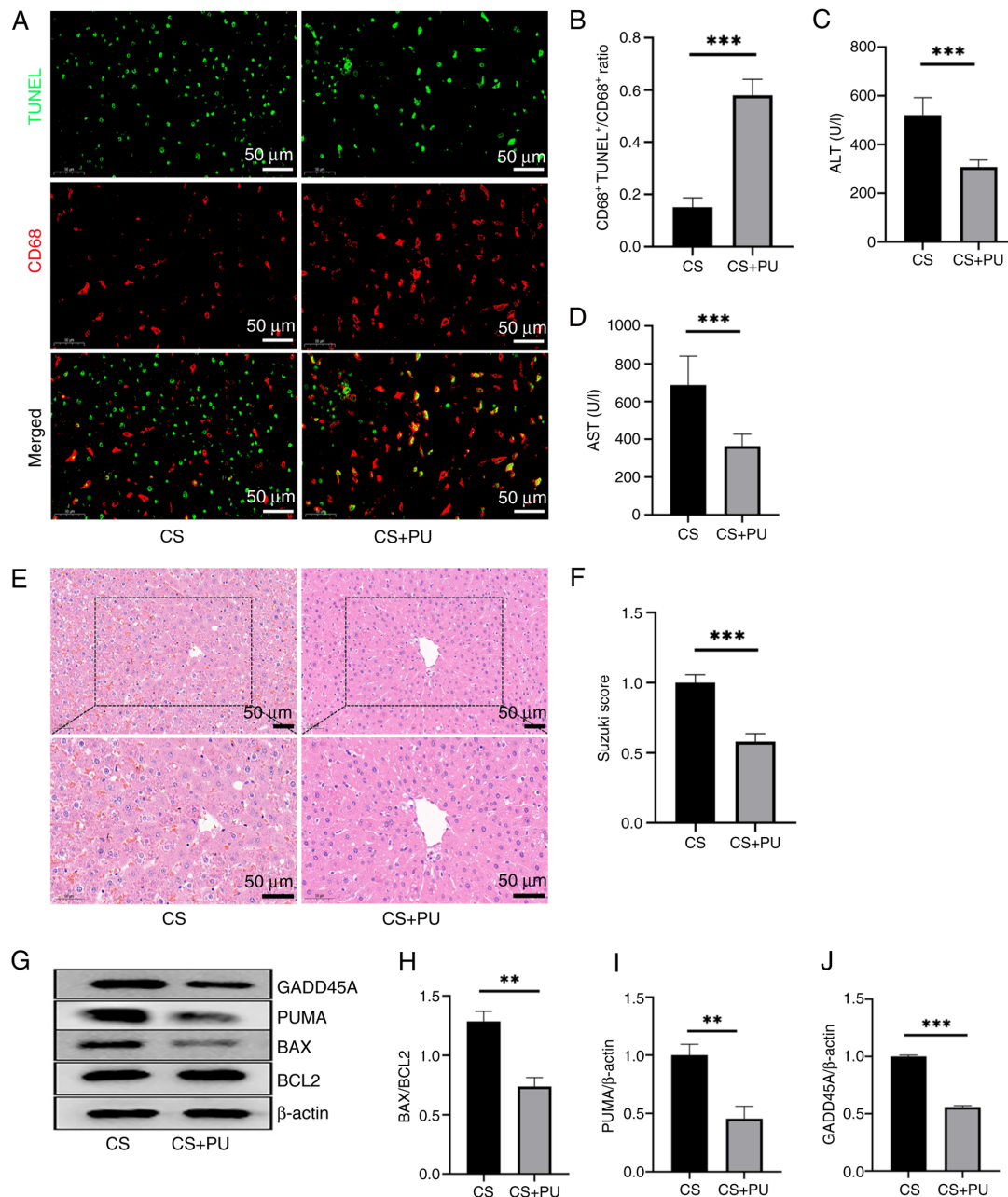


Figure 6. Increased macrophage efferocytosis improves the quality of donation after circulatory death donor livers. (A) Evaluation and (B) quantification of *in vivo* efferocytosis. Levels of (C) ALT and (D) AST in serum. (E) Hematoxylin and eosin staining and (F) Suzuki score in rat liver tissue (n=6). (G) Expression levels of (H) BCL2, BAX, (I) PUMA and (J) GADD45A. **P<0.01, ***P<0.001. AST, aspartate transferase; ALT, alanine transferase; PUMA, P53 upregulated modulator of apoptosis; GADD45A, growth arrest and DNA damage-inducible α ; CS, cold storage; PU, Putrescine.

macrophage efferocytosis (32,39). PU was administered to experimental animals and livers from DCD donors were preserved by CS. These livers were compared with those from conventional DCD donors that were not treated with PU to investigate whether increased efferocytosis events enhanced the protection of DCD livers. In the CS + PU group, efferocytosis events occurred more frequently than in the CS group (Fig. 6A and B). Assessment of liver function indicators and histopathological analysis demonstrated a significant attenuation of hepatocellular injury in the CS + PU group, as evidenced by decreased serum transaminase levels and reduced histopathological scores (Fig. 6C-F). Western blot analysis of BCL2, BAX, PUMA, and GADD45A indicated that enhanced efferocytosis reduced hepatocellular apoptosis

in DCD livers. This was evidenced by a downregulation of pro-apoptotic proteins (BAX, PUMA, and GADD45A) and a concomitant increase in the anti-apoptotic BCL2/BAX ratio, collectively shifting the protein expression profile toward an anti-apoptotic phenotype. (Fig. 6G-J). These findings suggested that promoting efferocytosis events improved the IRI of DCD livers. Moreover, the specific MerTK inhibitor UNC2025 significantly attenuated the effects of HMP on graft preservation and inflammatory response (Fig. S1A-D). In summary, the concurrent reduction in serum transaminase levels, improvement in histopathological scores, and decrease in apoptosis alongside enhanced efferocytosis in DCD liver grafts collectively demonstrate that the therapeutic efficacy of HMP depends on intact efferocytic function.

KLF2 inhibits the NLRP3-mediated pyroptosis pathway during CS/Rep in ECs. To investigate how KLF2 affects macrophage phagocytosis under CS/Rep conditions, RNA-seq was performed on the ov-control and the ov-KLF2 HUVECs after CS/Rep treatment. RNA-seq analysis revealed that 1,061 genes were up- and 1,584 genes were downregulated (Fig. 7A). GO enrichment analysis showed that these DEGs were primarily enriched in biological processes such as ‘angiogenesis’, ‘positive regulation of apoptotic process’, ‘cell-cell signaling’ and ‘inflammatory response’ (Fig. S2), suggesting that KLF2 overexpression significantly modulates inflammation- and immune-associated pathways during cold IRI in HUVECs.

It is known that the inflammatory microenvironment of macrophages influences their phenotypical characteristics and phagocytic function (40). The present differential gene analysis focused on the expression of NLRP3 (Fig. 7B) as NLRP3-mediated pyroptosis amplifies the inflammatory response (41). In addition to NLRP3, RNA-seq revealed significant changes in the expression of other genes associated with inflammation. Specifically, levels of pro-inflammatory factors such as IL-6 and JUN were significantly downregulated. NLRP3 expression levels were significantly lower in the ov-KLF2 compared with the ov-control group (Fig. 7C and D). To investigate the potential interaction between KLF2 and NLRP3, Co-IP was performed. There was a specific physical interaction between KLF2 and NLRP3, as evidenced by the presence of NLRP3 in the KLF2 immunoprecipitate and KLF2 in the NLRP3 immunoprecipitate (Fig. 7E). TEM was performed to observe signs of pyroptosis following CS/Rep treatment. Compared with the ov-control group, the ov-KLF2 group maintained relatively intact cell membranes. By contrast with the sh-control group, the sh-KLF2 group exhibited notable mitochondrial swelling with loss of most cristae, characteristic of pyroptosis (Fig. 7F). Western blot analysis of pyroptosis-associated molecules such as GSDMD, Caspase-1 and IL-18 (42–46) was performed. Overexpression of KLF2 significantly decreased the expression of NLRP3 and pyroptosis-related proteins, while the sh-KLF2 group showed the opposite trend (Fig. 7G–Q). Compared with the sh-KLF2 group, the sh-KLF2 + MCC950 group showed a significant inhibition in the expression of key pyroptosis-related proteins (Fig. S3A–E). These results collectively indicated that KLF2 upregulation suppressed the NLRP3-mediated pyroptosis pathway in ECs during CS/Rep. NLRP3 inhibitor under KLF2-deficient conditions effectively rescued the exacerbated pyroptotic phenotype.

EC pyroptosis-induced inflammation inhibits macrophage efferocytosis. To explore the impact of EC pyroptosis on macrophage efferocytosis, pyroptosis of ECs was altered using the pyroptosis agonist nigericin (Fig. 8A). As the dose of nigericin increased, HUVECs exhibited distinct morphological changes. The cells shrank or swelled, the outlines became blurred, the cytoplasm appeared sparse or expanded and structure became irregular (Fig. 8B). To assess how EC pyroptosis affect macrophages efferocytosis, ECs were co-cultured with macrophages for 24 h in a non-contact setting (Fig. 8C). Macrophages were co-incubated with ACs for 45 min. The efferocytosis capacity of macrophages was quantified based on the co-localization of the tracers of the two cell types. When the degree of EC

pyroptosis increased, macrophages co-cultured with these cells demonstrated a decreased ability to phagocytose apoptotic cells (Fig. 8D and E). Therefore, increased pyroptosis of ECs impaired macrophage efferocytosis.

Discussion

The present study investigated the protective crosstalk between ECs and macrophage efferocytosis during HMP for DCD livers. The present study confirmed the key role of endothelial KLF2 in DCD livers. Additionally, conventional static CS significantly downregulated KLF2 expression in LSECs, which was associated with impaired macrophage efferocytosis. Mechanistically, KLF2 negatively regulated the pyroptosis signaling pathway by directly interacting with NLRP3 in ECs, thereby attenuating the inflammatory microenvironment. This decrease facilitated macrophage efferocytosis. These findings demonstrate the regulatory role of KLF2 in the NLRP3 signaling cascade in ECs, underscoring the potential therapeutic value of targeting KLF2 in HMP to improve macrophage efferocytosis and counteract the impaired immune response during DCD liver storage.

Optimizing organ preservation methods serves a key role in minimizing IRI, especially in DCD livers. ECs, which line blood vessels, are vital integrators and sensors of physiological stimuli (47), actively participating in numerous processes by responding to changes in hemodynamic forces. Under physiological conditions, endothelial cells respond to changes in hemodynamic forces to maintain circulatory homeostasis. Under pathological conditions, however, this responsive mechanism becomes dysregulated, thereby promoting the development of vascular diseases such as hypertension, thrombosis, aneurysm, and atherosclerosis (48). In conventional static CS, the absence of biomechanical stimulation on the blood vessel walls results in damage to ECs (10,49–51), negatively impacting graft recovery. HMP is an advanced technology applied in organ preservation and transplantation. By mimicking physiological conditions in a hypothermic environment to match organ metabolic demands, HMP enhances physiological adaptability and increases the success rate of transplantation (52,53). HMP effectively alleviates EC damage caused by the lack of biomechanical stimulation during CS by providing flow stimulation, thereby improving EC function (13,54). A key mechanism underlying this positive effect is the upregulation of KLF2, a critical molecule that induces endothelial shear stress. KLF2 regulates endothelial gene expression, which is key for processes such as angiogenesis and the maintenance of vascular endothelial health under flow-induced shear stress (55). KLF2 influences the inflammatory response (56), angiogenesis (57) and antioxidant (58) defense in blood vessels by regulating the gene expression of ECs. Here, conventional static CS significantly downregulated KLF2 expression in LSECs, whereas HMP markedly upregulated its expression. Parallel plate flow chamber experiments *in vitro* confirmed that KLF2 served as a key effector molecule produced by vascular ECs in response to LSS stimulation. Moreover, HUVECs overexpressing KLF2 exhibited significantly decreased apoptosis following CS/Rep injury, which is consistent with previous studies (12,59). KLF2 is a key factor in maintaining the functional properties and homeostasis of

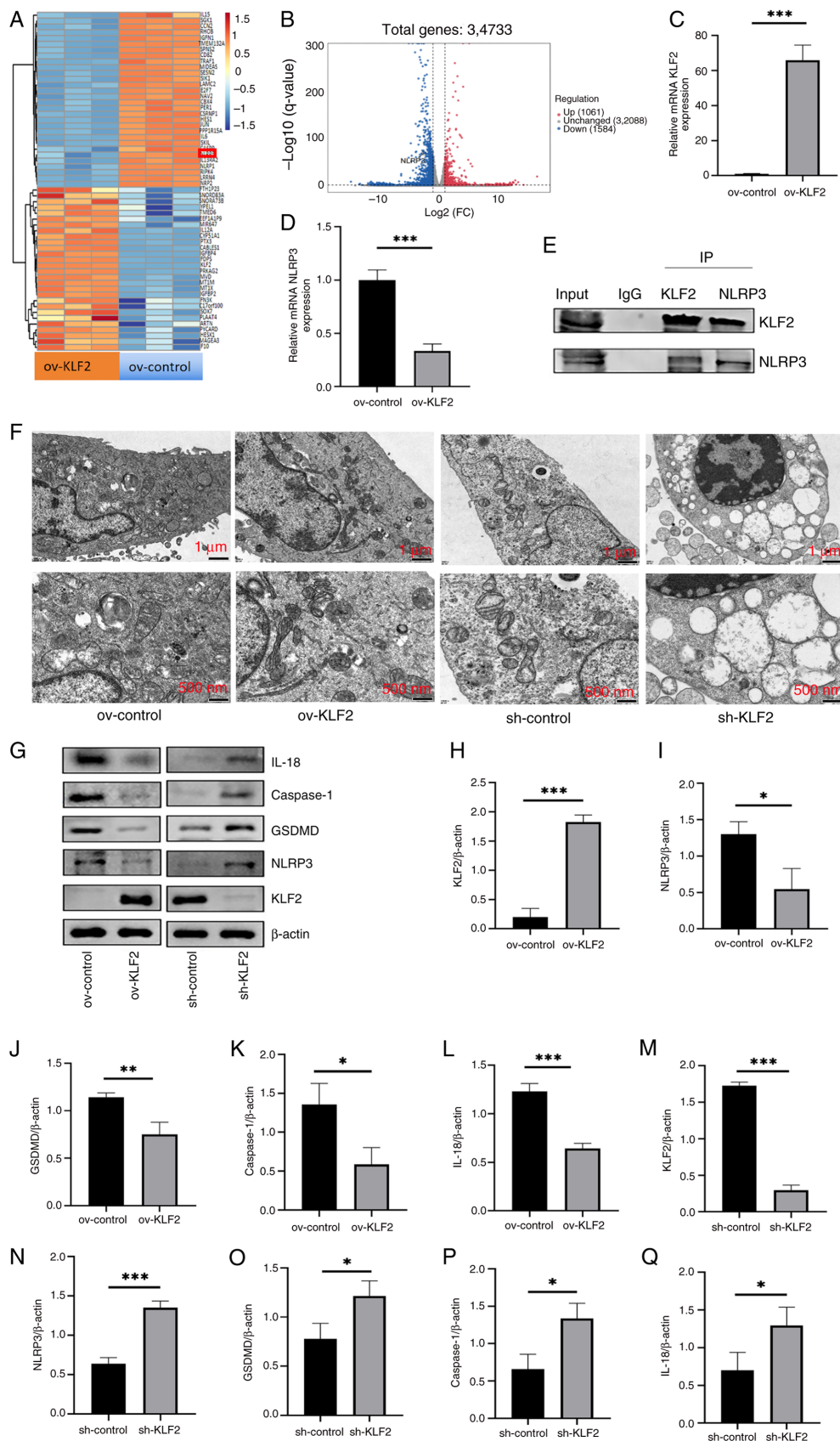


Figure 7. KLF2 inhibits the NLRP3-mediated pyroptosis pathway during CS/Rep in ECs. (A) Heatmap and (B) volcano plot of differentially expressed genes between control and ov-KLF2 HUVECs after CS/Rep treatment. mRNA levels of (C) KLF2 and (D) NLRP3 in ov-control and ov-KLF2 group of HUVECs following CS/Rep treatment were quantified using reverse transcription-quantitative PCR. (E) Co-IP analysis of the interaction between KLF2 and NLRP3. (F) Microstructure of HUVECs. (G) Representative Western blot images for KLF2, NLRP3, GSDMD, Caspase-1 and IL-18 in CS/Rep HUVECs. Quantitative analysis of KLF2 (H), NLRP3 (I), GSDMD (J), Caspase-1 (K) and IL-18 (L) protein expression based on western blot results from HUVECs of ov-control and ov-KLF2 groups. Quantitative analysis of KLF2 (M), NLRP3 (N), GSDMD (O), Caspase-1 (P) and IL-18 (Q) protein expression based on western blot results from HUVECs of sh-control and sh-KLF2 groups. (n=3). *P<0.05, **P<0.01, ***P<0.001. KLF2, Kruppel-like Factor 2; CS/Rep, cold storage/reperfusion; HUVEC, human umbilical vein endothelial cells; ov, overexpression; IP, immunoprecipitation; GSDMD, gasdermin D; sh, short hairpin; FC, fold-change.

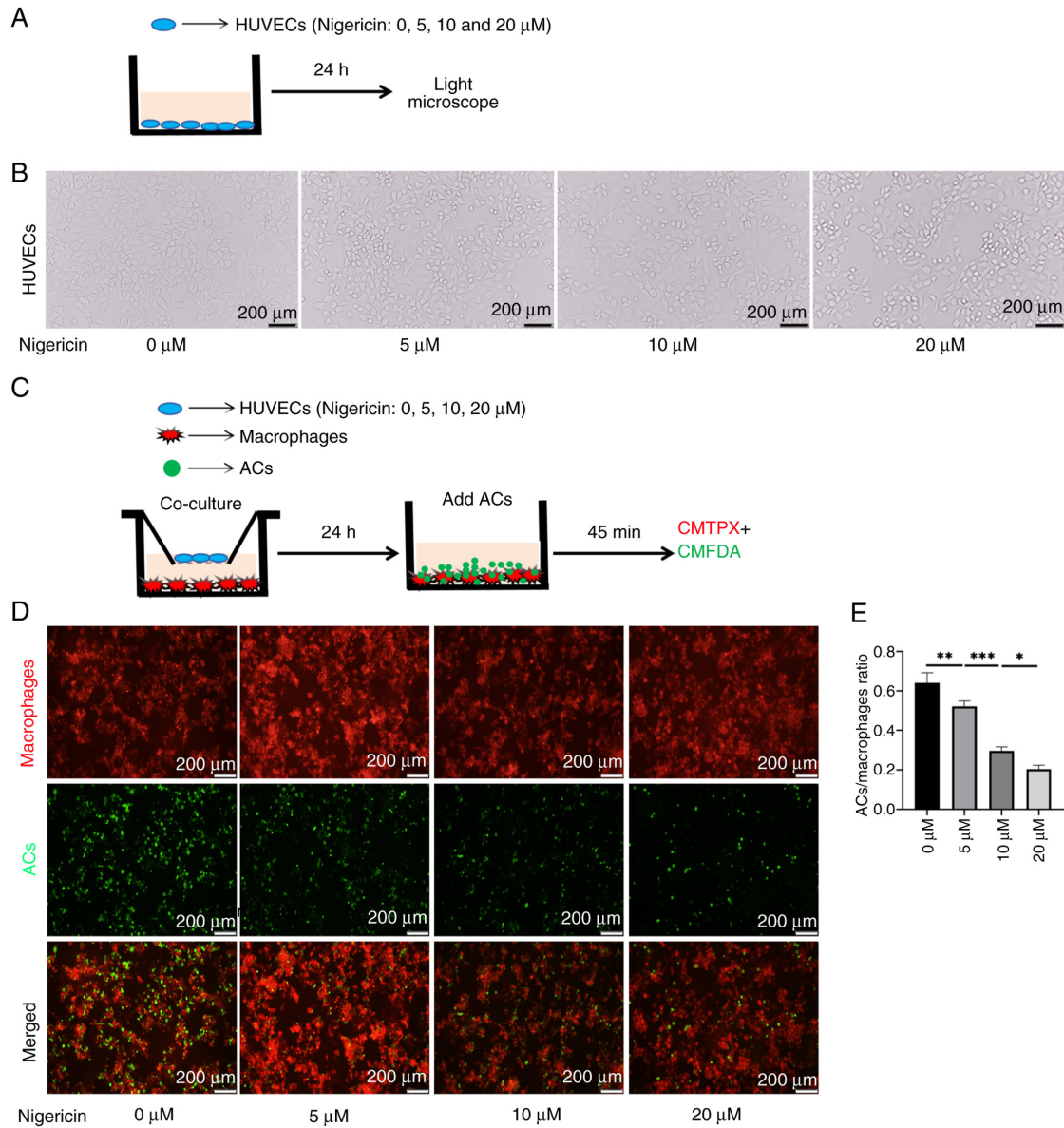


Figure 8. Pyroptosis of ECs inhibits the occurrence of efferocytosis in macrophages. (A) Experimental protocol of nigericin treatment. (B) Morphology and structure of HUVECs treated with cell pyroptosis agonist nigericin were investigated by bright-field imaging (n=3). (C) Schematic diagram of the procedure for adding nigericin to the HUVEC-macrophage co-culture system. (D) Representative staining images showing efferocytosis in macrophages underwent non-contact co-culture with HUVECs treated with nigericin. (E) Quantitative analysis of efferocytosis in macrophages. (n=3). *P<0.05, **P<0.01, ***P<0.001. AC, apoptotic Jurkat cell; HUVEC, human umbilical vein endothelial cell; CMTPX, CellTracker™ Red CMTPX dye; CMFDA, 5-Chloromethylfluorescein Diacetate.

ECs in a shear stress environment, highlighting its vital role in optimizing organ preservation and improving transplant outcomes. However, the specific role of HMP by elevating KLF2 expression in vascular ECs to improve DCD liver injury remains unclear.

In the present EC CS/Rep model, KLF2 exerted a potent anti-inflammatory effect by inhibiting the activation of the NLRP3 inflammasome. The NLRP3 protein belongs to the nucleotide-binding oligomerization domain-like receptor family (60). The assembly of the NLRP3 inflammasome is the canonical upstream event for caspase-1 activation. Detection of caspase-1 activation provides direct evidence of functional inflammasome activity and represents a key hallmark distinguishing the canonical pyroptosis pathway. GSDMD, following cleavage by caspase-1, forms pores in the plasma membrane, representing the definitive downstream step in pyroptosis

execution. Mature IL-18 serves as a key substrate of caspase-1. Its secretion not only validates caspase-1 activity but also directly explains the inflammatory response driven by pyroptosis. As a pattern recognition receptor, its key function is to assemble the NLRP3 inflammasome. NLRP3 has the broadest functional range of all inflammasomes in both the innate and adaptive immune systems (61,62), and its dysregulation is the root cause of numerous inflammatory diseases (63,64). For example, the pathogenesis of various immune- and inflammation-related diseases, such as including arthritis, Alzheimer's disease, inflammatory bowel disease, and other autoimmune or autoinflammatory disorders-is closely linked to the function of the NLRP3 inflammasome (65). Downregulating the NLRP3 inflammasome can exert a protective effect against liver IRI (33,66,67). ECs at inflammatory sites act as both active participants and regulators of the inflammatory

process (68). Studies (68,69) have confirmed that activation of the NLRP3 inflammasome in ECs exacerbates endothelial dysfunction. This activation triggers the NLRP3 inflammasome, leading to the secretion of mature forms of IL-1 β and IL-18, thereby intensifying the inflammatory response in ECs (70). This aligns with the present findings: Compared with the sh-control group, ECs with sh-KLF2, exhibited increased NLRP3 expression following CS/Rep, along with upregulation of NLRP3 inflammasome-associated proteins. Correspondingly, apoptosis levels in ECs were significantly elevated. sh-KLF2-transfected cells were treated with the specific NLRP3 inhibitor MCC950, followed by CS/Rep. The expression levels of pyroptosis-associated proteins were then assessed by western blotting. Compared with the sh-KLF2 group, MCC950 attenuated the increased expression of pyroptosis-related proteins induced by sh-KLF2, suggesting NLRP3 inhibition rescued the pyroptosis phenotype resulting from KLF2 deficiency. Mechanistically, KLF2 inhibits the activation of the NLRP3 inflammasome, decreases pyroptosis and ameliorates endothelial dysfunction. Additionally, NLRP3 inflammasome activation releases damage-associated molecular patterns such as high mobility group box 1 protein (HMGB1) into the extracellular environment (71). The inflammatory molecules released by NLRP3 inflammasome-mediated pyroptosis directly shape a persistently pro-inflammatory microenvironment. This microenvironment is associated with the regulation of macrophage efferocytosis; multiple inflammatory mediators, including HMGB1 (19) and IL-10 (16), modulate macrophage efferocytosis.

The present study not only observed enhanced efferocytosis but also demonstrated, through co-culture experiments, that endothelial KLF2 status regulated macrophage function. Efferocytosis serves as a key hub linking innate immunity and tissue repair, playing a key role in physiological and pathological processes by regulating the clearance of apoptotic cells (72,73). Macrophages efficiently remove apoptotic cells, preventing secondary necrosis and the release of inflammatory factors, thereby maintaining tissue homeostasis. Moreover, this process promotes macrophage polarization toward an anti-inflammatory and reparative (M2) phenotype, characterized by the release of anti-inflammatory cytokines such as IL-10 and TGF- β , which suppress excessive inflammatory responses (74). In the liver, efferocytosis serves a central role in maintaining homeostasis and suppressing inflammatory responses. In non-alcoholic steatohepatitis, impaired efferocytosis leads to the accumulation of apoptotic hepatocytes, exacerbating inflammation and fibrosis (75). By contrast, during liver IRI, efferocytosis exhibits notable anti-inflammatory and protective effects (16,76). This mechanism explains why the HMP group demonstrated milder inflammatory responses and enhanced tissue repair compared with CS group.

Nevertheless, the present study has several important limitations. First, the precise molecular mechanisms and signaling pathways by which pyroptosis in LSECs influences macrophage efferocytosis via paracrine signals remain incompletely elucidated. Future investigations should employ a multi-dimensional approach to explore this cascade: At the molecular level, identifying key mediators released by ECs following pyroptosis is key; at the cellular level, establishing more sophisticated co-culture systems is needed to identify

intercellular communication and at the tissue level, techniques such as spatial transcriptomics could be leveraged to visualize the signaling networks *in situ*.

Due to technical and time constraints, the present study did not conduct *in vivo* endothelial-specific KLF2 loss-of-function experiments to validate the role of KLF2 in liver protection. However, such investigations should be performed in future research. The present study demonstrated through rescue experiments that NLRP3 served as a key downstream mediator of KLF2-driven cytoprotection and efferocytosis. However, a complementary gain-of-function test (activating NLRP3 in a KLF2-overexpression setting) was not performed, which undermines the robustness of the conclusion. Future work should address this to delineate the KLF2-NLRP3 inhibitory axis. Furthermore, functional validation of TIMD4 in efferocytosis is limited by the lack of specific, well-characterized inhibitors or blocking antibodies, precluding direct loss-of-function evidence for TIMD4-dependent efferocytosis as a key phenotypical mediator. To address the broader role of efferocytosis in the observed phenotype, the present study performed loss-of-function experiments using a specific inhibitor of MerTK, another key efferocytosis receptor. PU, a central molecule in polyamine metabolism, participates broadly in physiological processes including cell proliferation and inflammation regulation (77). The pro-efferocytosis effect may reflect one facet of its pleiotropic functions rather than a specific agonist activity.

Second, the rat DCD model used in the present study presents limitations for clinical translation. The model lacks long-term functional assessment metrics post-transplantation and the short observation window precludes insights into long-term outcomes. Furthermore, the standardized experimental setting fails to recapitulate the donor-recipient heterogeneity inherent in clinical practice, such as variations in age, underlying comorbidities and immune status, which limit the direct extrapolation of the present findings to the clinical realm.

Notwithstanding these limitations, the proposed KLF2-NLRP3-efferocytosis axis has a well-defined trajectory for clinical translation. In the context of organ preservation, incorporating KLF2 agonists or NLRP3 inhibitors into machine perfusion solutions represents a promising *ex vivo* strategy to improve the quality of marginal donor livers. For transplant assessment, key molecular components of this axis, including KLF2, NLRP3 and efferocytosis markers, exhibit potential as novel biomarkers to predict post-transplant rejection and functional recovery. Development of integrated therapeutic strategies based on this mechanism (donor preconditioning, intraoperative organ protection and postoperative immunomodulation in recipients) may establish a novel treatment paradigm for DCD liver transplantation.

The present study highlighted the role of HMP in regulating the KLF2-derived transcriptional program in LSECs, contributing to vascular protection and promoting macrophage efferocytosis. Overexpression of KLF2 in ECs activated a KLF2/NLRP3-mediated pyroptosis paracrine mechanism that influenced macrophage efferocytosis, shaping the inflammatory microenvironment. The present study deepens the understanding of how HMP protects DCD donor livers, with implications for developing therapeutic strategies aimed at

improving macrophage function and mitigating liver IRI. Targeting ECs to regulate macrophage efferocytosis may offer a promising approach for enhancing liver preservation and recovery.

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Availability of data and materials

The data generated in the present study may be found in the Gene Expression Omnibus database under accession number GSE296118 or at the following URL: ncbi.nlm.nih.gov/gds/?term=GSE296118.

Authors' contributions

QD performed experiments, analyzed data and wrote and revised the manuscript. ZLi and QY designed the methodology and analyzed data. JL, XZ and ZF analyzed data. JL, ZLu and PY analyzed data and supervised the study. JX and QX designed and conceived the study and revised the manuscript. All authors have read and approved the final manuscript. QD and ZLi confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee for Laboratory Animal Welfare of the First Affiliated Hospital of Nanchang University (approval no. CDYFY-IACUC-202407QR199, Nanchang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Sugawara Y and Hibi T: Recent trends and new developments in liver transplantation. *Biosci Trends* 18: 206-211, 2024.
- Saidi RF and Hejazii Kenari SK: Challenges of organ shortage for transplantation: Solutions and opportunities. *Int J Organ Transplant Med* 5: 87-96, 2014.
- Hahn JW, Woo S, Park J, Lee H, Kim HJ, Ko JS, Moon JS, Rahmati M, Smith L, Kang J, *et al*: Global, regional, and national trends in liver Disease-related mortality across 112 countries from 1990 to 2021, with projections to 2050: Comprehensive analysis of the WHO mortality database. *J Korean Med Sci* 39: e292, 2024.
- Monbaliu D, Pirenne J and Talbot D: Liver transplantation using donation after cardiac death donors. *J Hepatol* 56: 474-485, 2012.
- Yue P, Lv X, You J, Zou Y, Luo J, Lu Z, Cao H, Liu Z, Fan X and Ye Q: Hypothermic oxygenated perfusion attenuates DCD liver ischemia-reperfusion injury by activating the JAK2/STAT3/HAX1 pathway to regulate endoplasmic reticulum stress. *Cell Mol Biol Lett* 28: 55, 2023.
- Czigany Z, Craigie EC, Lurje G, Song S, Yonezawa K, Yamamoto Y, Minor T and Tolba RH: Adenosine A2a receptor stimulation attenuates Ischemia-reperfusion injury and improves survival in A porcine model of DCD liver transplantation. *Int J Mol Sci* 21: 6747, 2020.
- Sousa Da Silva RX, Weber A, Dutkowski P and Clavien PA: Machine perfusion in liver transplantation. *Hepatology* 76: 1531-1549, 2022.
- Feng GY, Feng X, Tao J, Ao YP, Wu XH, Qi SG, He ZB and Shi ZR: Benefits of hypothermic oxygenated perfusion versus static cold storage in liver transplant: A comprehensive systematic review and Meta-analysis. *J Clin Exp Hepatol* 14: 101337, 2024.
- Parmar KM, Larman HB, Dai G, Zhang Y, Wang ET, Moorthy SN, Kratz JR, Lin Z, Jain MK, Gimbrone MA Jr and García-Cardena G: Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. *J Clin Invest* 116: 49-58, 2006.
- Gracia-Sancho J, Villarreal G Jr, Zhang Y, Yu JX, Liu Y, Tullius SG and García-Cardena G: Flow cessation triggers endothelial dysfunction during organ cold storage conditions: Strategies for pharmacologic intervention. *Transplantation* 90: 142-149, 2010.
- Russo L, Gracia-Sancho J, García-Calderó H, Marrone G, García-Pagán JC, García-Cardena G and Bosch J: Addition of simvastatin to cold storage solution prevents endothelial dysfunction in explanted rat livers. *Hepatology* 55: 921-930, 2012.
- Hu X, Wang W, Zeng C, He W, Zhong Z, Liu Z, Wang Y and Ye Q: Appropriate timing for hypothermic machine perfusion to preserve livers donated after circulatory death. *Mol Med Rep* 22: 2003-2011, 2020.
- Burlage LC, Karimian N, Westerkamp AC, Visser N, Matton APM, van Rijn R, Adelmeijer J, Wiersema-Buist J, Gouw ASH, Lisman T and Porte RJ: Oxygenated hypothermic machine perfusion after static cold storage improves endothelial function of extended criteria donor livers. *HPB (Oxford)* 19: 538-546, 2017.
- Liu Z, Zhang X, Xiao Q, Ye S, Lai CH, Luo J, Huang X, Wang W, Zeng C, Zhong Z, *et al*: Pretreatment donors after circulatory death with simvastatin alleviates liver ischemia reperfusion injury through a KLF2-dependent mechanism in rat. *Oxid Med Cell Longev* 2017: 3861914, 2017.
- Doran AC, Yurdagül A Jr and Tabas I: Efferocytosis in health and disease. *Nat Rev Immunol* 20: 254-267, 2020.
- Ni M, Zhang J, Sosa R, Zhang H, Wang H, Jin D, Crowley K, Naini B, Reed FE, Busuttill RW, *et al*: T-Cell immunoglobulin and mucin Domain-containing Protein-4 is critical for kupffer cell homeostatic function in the activation and resolution of liver ischemia reperfusion injury. *Hepatology* 74: 2118-2132, 2021.
- Miao L, Yu C, Guan G, Luan X, Jin X, Pan M, Yang Y, Yan J, Chen P and Di G: Extracellular vesicles containing GAS6 protect the liver from ischemia-reperfusion injury by enhancing macrophage efferocytosis via MerTK-ERK-COX2 signaling. *Cell Death Discov* 10: 401, 2024.
- Mihaila AC, Ciortan L, Tucureanu MM, Simionescu M and Butoi E: Anti-Inflammatory neutrophils reprogram macrophages toward a Pro-healing phenotype with increased efferocytosis capacity. *Cells* 13: 208, 2024.
- Pisetsky DS: The role of HMGB1 in efferocytosis: When the dead go unburied. Focus on 'HMGB1 inhibits macrophage activity in efferocytosis through binding to the α v β 3-integrin'. *Am J Physiol Cell Physiol* 299: C1253-C1255, 2010.
- Zhang J, Muri J, Fitzgerald G, Gorski T, Gianni-Barrera R, Masschelein E, D'Hulst G, Gilardoni P, Turiel G, Fan Z, *et al*: Endothelial lactate controls muscle regeneration from ischemia by inducing M2-like macrophage polarization. *Cell Metab* 31: 1136-1153.e7, 2020.
- Zhou B, Magana L, Hong Z, Huang LS, Chakraborty S, Tsukasaki Y, Huang C, Wang L, Di A, Ganesh B, *et al*: The angiocrine Rspodin3 instructs interstitial macrophage transition via metabolic-epigenetic reprogramming and resolves inflammatory injury. *Nat Immunol* 21: 1430-1443, 2020.
- Li C, Fang F, Wang E, Yang H, Yang X, Wang Q, Si L, Zhang Z and Liu X: Engineering extracellular vesicles derived from endothelial cells sheared by laminar flow for anti-atherosclerotic therapy through reprogramming macrophage. *Biomaterials* 314: 122832, 2025.

23. He S, Wu C, Xiao J, Li D, Sun Z and Li M: Endothelial extracellular vesicles modulate the macrophage phenotype: Potential implications in atherosclerosis. *Scand J Immunol* 87: e12648, 2018.
24. Ni CW, Qiu H, Rezvan A, Kwon K, Nam D, Son DJ, Visvader JE and Jo H: Discovery of novel mechanosensitive genes in vivo using mouse carotid artery endothelium exposed to disturbed flow. *Blood* 116: e66-e73, 2010.
25. Nam D, Ni CW, Rezvan A, Suo J, Budzyn K, Llanos A, Harrison D, Giddens D and Jo H: Partial carotid ligation is a model of acutely induced disturbed flow, leading to rapid endothelial dysfunction and atherosclerosis. *Am J Physiol Heart Circ Physiol* 297: H1535-H1543, 2009.
26. Lv JJ, Wang H, Zhang C, Zhang TJ, Wei HL, Liu ZK, Ma YH, Yang Z, He Q, Wang LJ, *et al*: CD147 sparks atherosclerosis by driving M1 phenotype and impairing efferocytosis. *Circ Res* 134: 165-185, 2024.
27. Adkar SS and Leeper NJ: Efferocytosis in atherosclerosis. *Nat Rev Cardiol* 21: 762-779, 2024.
28. Kojima Y, Weissman IL and Leeper NJ: The role of efferocytosis in atherosclerosis. *Circulation* 135: 476-489, 2017.
29. Parmar KM, Nambudiri V, Dai G, Larman HB, Gimbrone MA Jr and García-Cardena G: Statins exert endothelial atheroprotective effects via the KLF2 transcription factor. *J Biol Chem* 280: 26714-26719, 2005.
30. Yao X, Liu Y, Mao M, Yang L, Zhan Q and Xiao J: Calorie restriction mimetic, resveratrol, attenuates hepatic ischemia and reperfusion injury through enhancing efferocytosis of macrophages via AMPK/STAT3/S1PR1 pathway. *J Nutr Biochem* 126: 109587, 2024.
31. Chen F, Xu W, Tang M, Tian Y, Shu Y, He X, Zhou L, Liu Q, Zhu Q, Lu X, *et al*: hnRNP A2B1 deacetylation by SIRT6 restrains local transcription and safeguards genome stability. *Cell Death Differ* 32: 382-396, 2025.
32. Yurdagul A Jr, Subramanian M, Wang X, Crown SB, Ilkayeva OR, Darville L, Kolluru GK, Rymond CC, Gerlach BD, Zheng Z, *et al*: Macrophage metabolism of apoptotic Cell-derived arginine promotes continual efferocytosis and resolution of injury. *Cell Metab* 31: 518-533.e10, 2020.
33. He W, Ye S, Zeng C, Xue S, Hu X, Zhang X, Gao S, Xiong Y, He X, Vivalda S, *et al*: Hypothermic oxygenated perfusion (HOPE) attenuates ischemia/reperfusion injury in the liver through inhibition of the TXNIP/NLRP3 inflammasome pathway in a rat model of donation after cardiac death. *FASEB J*: Jun 5, 2018 (Epub ahead of print). doi: 10.1096/fj.201800028RR.
34. Zeng X, Li M, Fan X, Xue S, Liang W, Fan Z, Zeng C, Fan L, Xiong Y, Wang Y and Ye Q: Hypothermic oxygenated machine perfusion alleviates donation after circulatory death liver injury through regulating P-selectin-dependent and -independent pathways in mice. *Transplantation* 103: 918-928, 2019.
35. Suzuki S, Toledo-Pereyra LH, Rodriguez FJ and Cejalvo D: Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. *Transplantation* 55: 1265-1272, 1993.
36. Hu Q, Chen H, Lan J, Chen Y, Liu Z, Xiong Y, Zhou W, Zhong Z and Ye Q: KLF10 induced by hypothermic machine perfusion alleviates renal inflammation through BIRC2/Noncanonical NF- κ B pathway. *Transplantation* 109: e273-e286, 2025.
37. Petrusca DN, Gu Y, Adamowicz JJ, Rush NI, Hubbard WC, Smith PA, Berdyshev EV, Birukov KG, Lee CH, Tudor RM, *et al*: Sphingolipid-mediated inhibition of apoptotic cell clearance by alveolar macrophages. *J Biol Chem* 285: 40322-40332, 2010.
38. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
39. Yurdagul A Jr, Kong N, Gerlach BD, Wang X, Ampomah P, Kuriakose G, Tao W, Shi J and Tabas I: ODC (Ornithine Decarboxylase)-Dependent putrescine synthesis maintains MerTK (MER Tyrosine-Protein Kinase) expression to drive resolution. *Arterioscler Thromb Vasc Biol* 41: e144-e159, 2021.
40. Wang Y, Zhang W, Xu Y, Wu D, Gao Z, Zhou J, Qian H, He B and Wang G: Extracellular HMGB1 impairs macrophage-mediated efferocytosis by suppressing the Rab43-controlled cell surface transport of CD91. *Front Immunol* 13: 767630, 2022.
41. Vasudevan SO, Behl B and Rathinam VA: Pyroptosis-induced inflammation and tissue damage. *Semin Immunol* 69: 101781, 2023.
42. Jiang K, Tu Z, Chen K, Xu Y, Chen F, Xu S, Shi T, Qian J, Shen L, Hwa J, *et al*: Gasdermin D inhibition confers antineutrophil-mediated cardioprotection in acute myocardial infarction. *J Clin Invest* 132: e151268, 2022.
43. Li S, Sun Y, Song M, Song Y, Fang Y, Zhang Q, Li X, Song N, Ding J, Lu M and Hu G: NLRP3/caspase-1/GSDMD-mediated pyroptosis exerts a crucial role in astrocyte pathological injury in mouse model of depression. *JCI Insight* 6: e146852, 2021.
44. Liang F, Zhang F, Zhang L and Wei W: The advances in pyroptosis initiated by inflammasome in inflammatory and immune diseases. *Inflamm Res* 69: 159-166, 2020.
45. Liu Y, Pan R, Ouyang Y, Gu W, Xiao T, Yang H, Tang L, Wang H, Xiang B and Chen P: Pyroptosis in health and disease: Mechanisms, regulation and clinical perspective. *Signal Transduct Target Ther* 9: 245, 2024.
46. Zhang X, Wang Z, Li X, Chen J, Yu Z, Li X, Sun C, Hu L, Wu M and Liu L: Polydatin protects against atherosclerosis by activating autophagy and inhibiting pyroptosis mediated by the NLRP3 inflammasome. *J Ethnopharmacol* 309: 116304, 2023.
47. Gimbrone MA Jr, Topper JN, Nagel T, Anderson KR and Garcia-Cardena G: Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann N Y Acad Sci* 902: 230-240, 2000.
48. Ando J and Yamamoto K: Hemodynamic forces, endothelial mechanotransduction, and vascular diseases. *Magn Reson Med* 31: 258-266, 2022.
49. Caldwell-Kenkel JC, Currin RT, Tanaka Y, Thurman RG and Lemasters JJ: Reperfusion injury to endothelial cells following cold ischemic storage of rat livers. *Hepatology* 10: 292-299, 1989.
50. Peralta C, Jiménez-Castro MB and Gracia-Sancho J: Hepatic ischemia and reperfusion injury: Effects on the liver sinusoidal milieu. *J Hepatol* 59: 1094-1106, 2013.
51. Upadhyaya GA, Topp SA, Hotchkiss RS, Anagli J and Strasberg SM: Effect of cold preservation on intracellular calcium concentration and calpain activity in rat sinusoidal endothelial cells. *Hepatology* 37: 313-323, 2003.
52. Knijff LWD, van Kooten C and Ploeg RJ: The effect of hypothermic machine perfusion to ameliorate Ischemia-Reperfusion injury in donor organs. *Front Immunol* 13: 848352, 2022.
53. Schlegel A, de Rougemont O, Graf R, Clavien PA and Dutkowski P: Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. *J Hepatol* 58: 278-286, 2013.
54. Chatauret N, Coudroy R, Delpech PO, Vandebrouck C, Hosni S, Scepi M and Hauet T: Mechanistic analysis of nonoxygenated hypothermic machine perfusion's protection on warm ischemic kidney uncovers greater eNOS phosphorylation and vasodilation. *Am J Transplant* 14: 2500-2514, 2014.
55. Hergenreider E, Heydt S, Tréguer K, Boettger T, Horrevoets AJ, Zeiher AM, Scheffer MP, Frangakis AS, Yin X, Mayr M, *et al*: Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nat Cell Biol* 14: 249-256, 2012.
56. Jha P and Das H: KLF2 in regulation of NF- κ B-Mediated immune cell function and inflammation. *Int J Mol Sci* 18: 2383, 2017.
57. Dekker RJ, Boon RA, Rondaij MG, Kragt A, Volger OL, Eldekerkamp YW, Meijers JC, Voorberg J, Pannekoek H and Horrevoets AJ: KLF2 provokes a gene expression pattern that establishes functional quiescent differentiation of the endothelium. *Blood* 107: 4354-4363, 2006.
58. Nayak L, Lin Z and Jain MK: 'Go with the flow': How Krüppel-like factor 2 regulates the vasoprotective effects of shear stress. *Antioxid Redox Signal* 15: 1449-1461, 2011.
59. Gallinat A, Efferz P, Paul A and Minor T: One or 4 h of 'in-house' preconditioning by machine perfusion after cold storage improve reperfusion parameters in porcine kidneys. *Transpl Int* 27: 1214-1219, 2014.
60. Xiao L, Magupalli VG and Wu H: Cryo-EM structures of the active NLRP3 inflammasome disc. *Nature* 613: 595-600, 2023.
61. Lamkanfi M and Dixit VM: Inflammasomes and their roles in health and disease. *Annu Rev Cell Dev Biol* 28: 137-161, 2012.
62. Zhang Y, Yang W, Li W and Zhao Y: NLRP3 Inflammasome: Checkpoint connecting innate and adaptive immunity in autoimmune diseases. *Front Immunol* 12: 732933, 2021.
63. Mangan MSJ, Olhava EJ, Roush WR, Seidel HM, Glick GD and Latz E: Targeting the NLRP3 inflammasome in inflammatory diseases. *Nat Rev Drug Discov* 17: 588-606, 2018.
64. Franchi L, Eigenbrod T, Muñoz-Planillo R and Núñez G: The inflammasome: A caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol* 10: 241-247, 2009.
65. Chen Y, Ye X, Escames G, Lei W, Zhang X, Li M, Li M, Jing T, Yao Y, Qiu Z, *et al*: The NLRP3 inflammasome: Contributions to inflammation-related diseases. *Cell Mol Biol Lett* 28: 51, 2023.

66. Dai J, Chen Q, Huang W, Shi K, Zhang Y, Li T, Mou T, Huang Z and Wu Z: Liver kinase B1 attenuates liver ischemia/reperfusion injury via inhibiting the NLRP3 inflammasome. *Acta Biochim Biophys Sin (Shanghai)* 53: 601-611, 2021.
67. Pu JL, Huang ZT, Luo YH, Mou T, Li TT, Li ZT, Wei XF and Wu ZJ: Fisetin mitigates hepatic ischemia-reperfusion injury by regulating GSK3 β /AMPK/NLRP3 inflammasome pathway. *Hepatobiliary Pancreat Dis Int* 20: 352-360, 2021.
68. Bai B, Yang Y, Wang Q, Li M, Tian C, Liu Y, Aung LHH, Li PF, Yu T and Chu XM: NLRP3 inflammasome in endothelial dysfunction. *Cell Death Dis* 11: 776, 2020.
69. Xu T, Chen T, Fang H, Shen X, Shen X, Tang Z and Zhao J: Human umbilical cord mesenchymal stem cells repair endothelial injury and dysfunction by regulating NLRP3 to inhibit endothelial cell pyroptosis in Kawasaki disease. *Inflammation* 47: 483-502, 2024.
70. Chen Z, Martin M, Li Z and Shyy JY: Endothelial dysfunction: The role of sterol regulatory element-binding protein-induced NOD-like receptor family pyrin domain-containing protein 3 inflammasome in atherosclerosis. *Curr Opin Lipidol* 25: 339-349, 2014.
71. Keyel PA: How is inflammation initiated? Individual influences of IL-1, IL-18 and HMGB1. *Cytokine* 69: 136-145, 2014.
72. Poon IKH and Ravichandran KS: Targeting efferocytosis in inflammaging. *Annu Rev Pharmacol Toxicol* 64: 339-357, 2024.
73. Boada-Romero E, Martinez J, Heckmann BL and Green DR: The clearance of dead cells by efferocytosis. *Nat Rev Mol Cell Biol* 21: 398-414, 2020.
74. Kourtzelis I, Hajishengallis G and Chavakis T: Phagocytosis of apoptotic cells in resolution of inflammation. *Front Immunol* 11: 553, 2020.
75. Shi H, Wang X, Li F, Gerlach BD, Yurdagul A Jr, Moore MP, Zeldin S, Zhang H, Cai B, Zheng Z, *et al*: CD47-SIRP α axis blockade in NASH promotes necroptotic hepatocyte clearance by liver macrophages and decreases hepatic fibrosis. *Sci Transl Med* 14: eabp8309, 2022.
76. Hu H, Cheng X, Li F, Guan Z, Xu J, Wu D, Gao Y, Zhan X, Wang P, Zhou H, *et al*: Defective efferocytosis by aged macrophages promotes STING signaling mediated inflammatory liver injury. *Cell Death Discov* 9: 236, 2023.
77. Pegg AE: Functions of polyamines in mammals. *J Biol Chem* 291: 14904-14912, 2016.



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