

# CKLF1 regulates ferroptosis in ischaemic-hypoxic cardiomyocytes through the Keap1/Nrf2/Ho1 signalling pathway

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## Article

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# Abstract

## Objective

To investigate the role of chemokine-like factor 1 (CKLF1) in cardiomyocyte ferroptosis in rats with acute myocardial infarction (AMI) and H9c2 cardiomyocytes subjected to oxygen-glucose deprivation (OGD).

## Methods

AMI was induced in rats via permanent ligation of the left anterior descending coronary artery, and an OGD model was established in H9c2 cells. Interventions included intramyocardial injection of a CKLF1-neutralizing antibody *in vivo* and application of the CKLF1 antagonist peptide C19 *in vitro*. ELISA was used to measure the levels of intracellular iron, glutathione (GSH), reactive oxygen species (ROS) and malondialdehyde (MDA) in serum and cell supernatant. Immunofluorescence, qPCR and Western blotting were used to detect the expression of the genes encoding glutathione peroxidase 4 (GPX4) and long-chain fatty acid CoA ligase 4 (ACSL4), and Western blotting was used to measure the expression of Keap1, Nrf2 and HO-1.

## Results

Administration of a CKLF1-neutralizing antibody reduced the infarct size in rats. In H9c2 cells, OGD-induced damage was mitigated by C19. Iron, ROS, GSH, and MDA levels in cardiomyocytes were altered after AMI and OGD, but reversible with CKLF1 neutralizing antibody or C19 treatment. Similarly, AMI and OGD upregulated ACSL4 and downregulated GPX4, effects also reversed by the respective treatments. The Keap1/Nrf2/HO-1 pathway was activated after AMI and OGD in H9c2 cells. ML385-mediated Nrf2 inhibition reversed this activation and altered GPX4 and ACSL4 expression. Similarly, C19 treatment restored the expression of this pathway and its downstream ferroptosis-related genes.

## Conclusion

CKLF1 promotes cardiomyocyte ferroptosis through the Nrf2/Keap1/HO-1 signalling pathway.

## Introduction

Acute myocardial infarction (AMI) remains a leading cause of global morbidity and mortality [1]. A severe inflammatory response is the first event of AMI. The infiltration of inflammatory cells and the release of inflammatory factors can promote the production and accumulation of free radicals and reactive oxygen species, leading to oxidative stress damage[2]. The inflammatory response and oxidative stress lead to cardiomyocyte necrosis, apoptosis, and autophagy. It is well known that adult mammalian

cardiomyocytes cannot regenerate; thus the greatest challenge in the treatment of myocardial infarction is to preventing cardiomyocyte death.

Chemokine-like factor 1 (CKLF1) was cloned and discovered by Chinese scientists. CKLF1 is a highly hydrophobic basic secretory protein with a molecular weight of 8–13 kDa and a typical molecular structure of the CC chemokine subfamily[3]. Current studies have shown that CKLF1 mainly plays a chemotactic role in the process of disease and participates in the inflammatory process[4, 5]. At the onset of AMI, a large number of chemokines and inflammatory cells initiate an inflammatory storm. As a member of the chemokine family, CKLF1 is likely involved in the pathological process of AMI in the inflammatory phase. In fact, our previous study confirmed the high expression of CKLF1 in myocardial infarction[6], suggesting that CKLF1 is an important target for the treatment of ischaemic injury, which provides support for our hypothesis.

Ferroptosis is an iron-dependent form of programmed cell death[7]. Iron metabolism, amino acid metabolism and lipid metabolism play important roles in the occurrence of ferroptosis[8]. Ferroptosis is involved in the occurrence and development of a variety of diseases[8–10]. Recent studies have shown that cardiomyocyte ferroptosis occurs during the pathogenesis of myocardial infarction. Hypoxia-reperfusion injury can induce myocardial ferroptosis, and inhibition of ferroptosis can reduce infarct size, reduce neutrophil infiltration, and improve postinfarction cardiac function and myocardial fibrosis[11–13]. Therefore, ferroptosis plays an important role in myocardial injury, but most studies have not elucidated its specific molecular mechanism, and the specific role of ferroptosis in AMI has not been studied. Therefore, clarifying the role of ferroptosis in AMI will have great research and clinical application value.

In conclusion, CKLF1 is a key regulator of ferroptosis in ischaemic and hypoxic cardiomyocytes, affecting iron metabolism, oxidative stress, and antioxidant defence mechanisms. Therefore, the present study was designed to elucidate the role of CKLF1 in protecting cardiomyocytes from ferroptosis in AMI, mainly through activation of the Keap1/Nrf2/HO-1 signalling pathway. Accordingly, we will verify the above conclusions from in vivo and in vitro experiments, clarify the underlying mechanism, and provide drug targets for the treatment of hypoxic-ischaemic cardiovascular diseases such as myocardial infarction.

## Materials and Methods

### Animals

Male Sprague-Dawley (SD) rats, weighing 250–280 g, were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The rats were housed in cages under controlled environmental conditions with a comfortable temperature of 22°C and a relative humidity of 40%–60%. The animals had free access to food and water throughout the study. All experimental procedures were conducted in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory

Animals and were approved by the Institutional Animal Care and Use Committee of university of South China (approval number: SYXK2022-0008).

### Induction of AMI and Precordial Injection in Rats

After anesthetizing the rats with 2% isoflurane, a tracheotomy was performed at the second and third tracheal rings. A tracheal tube was inserted and connected to a small animal ventilator. The ventilator's respiratory rate was set to 90/min, and the respiratory rate was set to 1:1. Anesthesia was maintained with 1.5% isoflurane, 70% N<sub>2</sub>O, and 30% O<sub>2</sub>. The left anterior wall of the heart was exposed, and a 6 – 0 suture was used to ligate the area 3–4 mm below the junction of the aortic arch and the left atrial appendage. The chest was then closed. The success of the AMI model was confirmed by electrocardiogram (ECG). In the sham-operated group, the suture was passed without ligation.

In the experiment, rats were randomly divided into three groups: the sham-operated group, the AMI group, and the AMI + CKLF1 neutralizing antibody group. In the AMI + CKLF1 neutralizing antibody group, after ligation of the left anterior descending (LAD) coronary artery, an insulin needle was used to inject the CKLF1 neutralizing antibody at four points in the precordial region supplied by the LAD. Each point was injected with 10 µl of the antibody. After injection, the chest was closed, and the rats were housed for a specified period for subsequent experiments. All groups of rats were euthanized with an intraperitoneal injection of pentobarbital sodium at a dose of 200mg/kg.

### Cells

Rat cardiomyocytes H9c2 were cultured in High-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C, 5% CO<sub>2</sub>. Cells were seeded at 1×10<sup>6</sup> cells /ml in six-well plates. The cells were divided into control group, oxygen-glucose deprivation (OGD) group, and OGD + antagonist peptide C19 group (1µM C19 was added) for 24 hours.

The model of OGD was established

After 12 hours, the cells in the OGD group and the antagonist group were replaced with nitrogen-blew, glucose-free Earle's solution. Then the cells in the OGD group and the antagonist group were placed in the hypoxia chamber. After the oxygen detector showed that the oxygen content was less than 0.5%, the air intake and outlet were quickly clamped. The hypoxia chamber was transferred to an incubator at 37°C and continued to be cultured for an appropriate time for subsequent experiments.

### Determination of GSH content

The content of GSH in the samples was determined by measuring the absorbance at 400nm using a microplate reader according to the instructions of the GSH kit.

### Determination of ROS content

After adding DCFH-DA, the samples were incubated at 37°C for 20min, and five non-repetitive areas were selected for photography under fluorescence microscope. The average green fluorescence intensity of the five fields was analyzed by ImageJ software, and then the statistical analysis was performed.

#### Determination of MDA content

After sample treatment, the protein was taken and quantified. The absorbance at 532nm was detected according to the instructions, and the MDA content was calculated.

#### Determination of intracellular iron

After the samples were added with the corresponding reagents according to the instructions, the absorbance at 593 nm was measured by microplate reader, and the intracellular iron content was calculated.

#### Immunofluorescence

Sections were fixed in 4% paraformaldehyde for 20min, then permeabilized with 0.5% Triton for 15min. Then 5% BSA was added to block for 30min at room temperature. Primary antibodies were added and incubated overnight in a 4°C refrigerator. The primary antibodies used were GPX4 (Abcam, 125066, 1:200) and ACSL4 (Abcam, 155282, 1:200). Rewarming was performed for 30min. Secondary antibodies and DAPI were incubated for 2h at room temperature in the dark. Anti-quench sealing tablets were sealed, dried, and stored at -20°C in the dark. Images were captured by laser confocal microscopy.

#### Real-time Quantitative PCR (qPCR)

Samples were lysed by adding 0.5ml Trizol. Add 200ul chloroform to each well, stir and mix, stand on ice for 10min, 4°C, 12000g, 6-stage deceleration centrifugation for 15min, draw 300-500ul of the supernatant into a clean centrifuge tube, add 500ul isopropanol, mix, place at -20°C for 10min, 4°C, 16000g centrifugation for 10min, discard the supernatant, 1 mL of 75% ethanol was added and the precipitate was washed at 4°C. The samples were centrifuged at 16000G for 5min, the supernatant was discarded, and 20 to 50µl DEPC water was added to dissolve the RNA. The concentration of the samples was detected by a microquantifier, and RNA with an absorbance of A260/A280 = 1.8–2.1 was selected for reverse transcription. Reverse cDNA was referred to the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit, and 1ug of RNA was used to reverse cDNA. The cDNA was amplified using an AB 7900HT real-time quantitative PCR instrument with reference to the TransStart Tip Green qPCR Supermix kit. PCR amplification conditions were as follows: 94°C, 30s; 40 cycles were performed at 94°C for 5s and 60°C for 30s. The experimental results were analyzed by the  $2^{-\Delta\Delta CT}$  method, and  $\beta$ -actin was used as the reference gene. GPX4: F-5'GCAACCAGTTTGGGAGGC AGGAG 3', R5'CCTCCATG GGACCATAGCGC TTC3'; ACSL4: F-5'CCCCAGACAC A CCGATTCA3', R-5'TTCCAGTAGTG TAGTCGGTAACTTC3';  $\beta$ -actin F-5'TCAGG TCATCACTAT CGGCAAT 3', R-5'AAAGAAAGGGTGTAAC GCA 3'

## Western blot analysis

Samples were added with 50ul of lysate in an ice bath for 30min, vortexed every 10min, centrifuged at 1000g at 4°C for 5min, and the supernatant was aspirated, which was the sample protein, and the protein concentration was determined by BCA quantification kit. Proteins were separated by 10%SDS-PAGE gel (40ug per sample) with 60V separation gel and 120V concentration gel. The separated proteins were transferred to PVDF membrane, blocked with 5%BSA, and the membrane was incubated with primary antibodies overnight at 4°C: Anti-GPX4 (abcam, ab125066,1:500), anti-ACSL4 (abcam, ab155282,1:500), anti-Nrf2 (abcam, ab62352,1:500), anti-Keap1 (abcam, ab166721,1:500), Anti-Ho1 (abcam, ab3052901:500), GADPH (1:1000) was used as an internal control. After washing three times with TBST, the cells were incubated with HRP-sheep anti-rabbit IgG secondary antibody or HRP-sheep anti-IgG secondary antibody for 2h at room temperature with shaking. The protein expression was detected by enhanced chemiluminescence detection system. Image J software was used to analyze the optical density values of the band at the target molecular weight and the internal reference band.

## Statistical Analysis

All data are expressed as Mean±standard error (Mean ± SEM) and analyzed using GraphPad Prism 7.0 (GraphPad Software, USA) data statistics software. In this study, the statistical method of One-way ANOVA with Turkey's test and two-way ANOVA with Bonferroni's multiple comparison test were selected. A P value of less than 0.05 was considered statistically significant.

## Results

### Ferroptosis is Activated in the Myocardial Infarct Zone Following Acute Myocardial Infarction in Rats

Recent studies have shown that GPX4 and ACSL4 play important roles in regulating the occurrence of ferroptosis [29]. To verify whether ferroptosis occurred after AMI in rats, qPCR and Western blotting were used to measure the expression of GPX4 and ACSL4 in the infarct area. The results revealed that GPX4 expression was decreased, whereas ACSL4 expression was significantly increased at 1, 3, and 7 days after surgery (Fig. 1A-E). Furthermore, immunofluorescence (Fig. 1F, G) revealed that the fluorescence intensity of GPX4 decreased and that of ACSL4 increased in myocardial cells in the infarct border zone at 1 and 7 days after surgery. These results suggest that ferroptosis occurs in rat cardiomyocytes during myocardial infarction.

### CKLF1-Neutralizing Antibody Attenuates Myocardial Infarction in Rat Models

The role of CKLF1 in myocardial infarction was assessed by neutralizing its activity in a rat model. Following ligation of the left anterior descending coronary artery, rats in the CKLF1 antibody group received an intramyocardial injection of the neutralizing antibody into the anterior wall. Rats in the AMI model group underwent ligation alone. The TTC staining results (Fig. 2A, B) revealed that the CKLF1 neutralizing antibody reduced infarct size in rats. Serum levels of CK-MB and LDH were reduced in rats

by a CKLF1-neutralizing antibody (Fig. 2C,D). These results indicate that CKLF1 neutralizing antibody can inhibit the occurrence of myocardial infarction in rats.

#### CKLF1-Neutralizing Antibody Attenuates CKLF1 Expression in the Infarcted Myocardium of Rats

Previous studies have demonstrated that CKLF1-neutralizing antibodies attenuate myocardial infarction in rats. Next, we aimed to investigate the effect of intramyocardial injection of CKLF1-neutralizing antibodies on CKLF1 expression. Compared with the AMI model group, the group with intramyocardial injection of a CKLF1-neutralizing antibody had dramatically reduced CKLF1 fluorescence intensity in the infarct area (Fig. 3A, B). qPCR and WB revealed that the anti-CKLF1 neutralizing antibody reduced CKLF1 expression in the infarct area (Fig. 3C-E). These findings suggest that administration of a CKLF1 neutralizing antibody decreases CKLF1 expression in the infarcted myocardium of rats.

#### CKLF1-Neutralizing Antibody Attenuates Cardiomyocyte Ferroptosis in the Infarcted Myocardium of Rats

Previous data have demonstrated that a CKLF1 neutralizing antibody can decrease CKLF1 expression in the infarcted area of rats; however, whether it can also suppress ferroptosis in cardiac cells in the infarcted area is unclear. The results demonstrated that (Fig. 4A-D) injection of a CKLF1-neutralizing antibody reduced the iron, ROS, and MDA contents while increasing the GSH content in the infarcted tissue. Preliminary evidence suggests that CKLF1-neutralizing antibodies can decrease cardiac cell ferroptosis. We next employed immunofluorescence and WB to confirm these conclusions by measuring the expression of the ferroptosis-related proteins GPX4 and ACSL4 in the infarct area.

Immunofluorescence staining revealed that the fluorescence intensity of GPX4 increased while the fluorescence intensity of ACSL4 decreased in infarct-area tissues injected with the CKLF1-neutralizing antibody compared with the AMI group (Fig. 4E-H). WB results demonstrated that, compared with the AMI group, the CKLF1-neutralizing antibody increased the expression of GPX4 in the infarction area while decreasing the expression of ACSL4 (Fig. 4I-K). These findings indicate that CKLF1 neutralizing antibodies can suppress ferroptosis in the infarct area.

#### The Keap1/Nrf2/Ho1 Signalling Pathway Was Activated After AMI in Rats

Nuclear Factor E2-related Factor 2 (Nrf2) plays a key role in regulating the expression of genes encoding antioxidant enzymes, thereby reducing ROS production and enhancing resistance to oxidative stress[14]. Moreover, Nrf2 has been shown to regulate ferroptosis in cells[15]. Therefore, we detected whether the Keap1/Nrf2/HO-1 signalling pathway was activated after AMI in rats. The WB results revealed that the protein expression of Keap1 in the infarction area of rats decreased after AMI, whereas the protein expression of Nrf2 and HO-1 increased. After the CKLF1 neutralizing antibody was injected into the infarcted myocardium, the expression of Keap1 increased compared with that in the AMI group, whereas the expression of Nrf2 and HO-1 decreased (Fig. 5A-D). Thus, the Keap1/Nrf2/HO-1 signalling pathway is activated in rats with AMI.

#### OGD Induces Ferroptosis in Cardiomyocytes

Previous investigations have shown that cardiomyocyte ferroptosis occurs in the infarcted area of a myocardial infarction in rats. Next, we investigated whether cardiomyocyte ferroptosis occurs following OGD. Therefore, we initially investigated ferroptosis-related factors in cardiomyocytes following OGD. After OGD, the levels of iron, ROS, and MDA in H9c2 cardiomyocytes increased, whereas the levels of GSH decreased (Fig. 6A-D). These findings suggest that cardiomyocytes undergo ferroptosis following OGD. Next, we examined the expression of the ferroptosis-related proteins GPX4 and ACSL4 in H9c2 cardiomyocytes after OGD. Immunofluorescence staining revealed that the fluorescence intensity of GPX4 in H9c2 cells significantly decreased after OGD, whereas the fluorescence intensity of ACSL4 increased (Fig. 6E-H). Furthermore, WB results indicated that the protein expression of GPX4 in H9c2 cells decreased after OGD, while the protein expression of ACSL4 increased (Fig. 6I-K). These results confirmed that ferroptosis occurred in rat cardiomyocytes after OGD and further demonstrated that cardiomyocytes in the infarction area after rat myocardial infarction could undergo ferroptosis.

### C19 Attenuates OGD-Induced Cardiomyocyte Injury and Suppresses CKLF1 Expression

To investigate the role of CKLF1 in cardiomyocyte ferroptosis, cardiomyocytes were treated with the CKLF1 antagonist peptide C19 prior to OGD. First, LDH release in the supernatant was measured to assess the effect of C19 on cardiomyocyte injury following OGD. Compared with the control treatment, 0.5  $\mu$ M, 1  $\mu$ M, and 2  $\mu$ M C19 reduced the LDH concentration in the H9c2 cardiomyocyte supernatant after OGD, indicating that C19 could reduce myocardial cell injury (Fig. 7A). C19 is a CKLF1 antagonistic peptide segment, and whether this affects CKLF1 expression remains unclear. Our earlier investigation demonstrated that after OGD, CKLF1 protein expression increased in cardiomyocytes[6]. Cardiomyocytes were subsequently treated with 1  $\mu$ M C19, and CKLF1 protein expression was evaluated by Western blot analysis 12 hours after OGD. The results revealed that C19 significantly attenuated OGD-induced upregulation of CKLF1 protein expression (Fig. 7B, C).

### C19 Suppresses Ferroptosis in Cardiomyocytes after OGD

Next, we studied the effect of C19 on the ferroptosis of cardiomyocytes after OGD. First, ELISA was used to detect the effects of C19 on ferroptosis-related factors in cardiomyocytes after OGD. The results revealed that 1  $\mu$ M C19 reversed the increase in iron, ROS and MDA contents and the decrease in GSH content in cardiomyocytes induced by OGD (Fig. 8A-D). Furthermore, the effects of C19 on the expression of GPX4 and ACSL4 induced by OGD in cardiomyocytes were detected by immunofluorescence, qPCR and WB to determine the role of CKLF1 in OGD-induced ferroptosis in cardiomyocytes. Immunofluorescence staining revealed that 1  $\mu$ M C19 significantly increased the fluorescence intensity of GPX4 attenuation induced by OGD, but decreased the fluorescence intensity of ACSL4 enhancement (Fig. 8E-H). The qPCR results revealed that 1  $\mu$ M C19 reversed the OGD-induced decrease in GPX4 mRNA expression in H9c2 cells and reversed the increase in ACSL4 mRNA expression (Fig. 8I, J). The WB results revealed that 1  $\mu$ M C19 reversed the OGD-induced decrease in GPX4 protein expression in H9c2 cells and reversed the increase in ACSL4 protein expression (Fig. 8K-M). These results confirmed that C19 inhibits ferroptosis induced by OGD in cardiomyocytes.

## CKLF1 Regulates Ferroptosis in H9c2 Cells after OGD through the Keap1/Nrf2/Ho1 Signalling Pathway

To investigate the role of the Keap1/Nrf2/HO-1 signalling pathway in ferroptosis during OGD, we examined its expression in H9c2 cells. Our results demonstrated that OGD significantly downregulated Keap1 protein expression while upregulating Nrf2 and Ho1 protein levels (Fig. 9A-D). To further elucidate the involvement of this pathway, we used the Nrf2 inhibitor ML385, which reversed the OGD-induced effects, leading to increased Keap1 expression and decreased Nrf2 and Ho1 levels compared with those in the OGD group (Fig. 9A-D). Notably, treatment with the CKLF1 inhibitor C19 counteracted these changes, restoring the expression profile of the Keap1/Nrf2/Ho1 pathway. These results indicate that CKLF1 can affect the activation of the Keap1/Nrf2/HO-1 signalling pathway in H9c2 cells after OGD.

To determine whether CKLF1 regulates ferroptosis through the Keap1/Nrf2/HO-1 pathway, we assessed key ferroptosis-related proteins, GPX4 and ACSL4. Compared with OGD alone, treatment with C19, ML385, or their combination resulted in increased GPX4 expression and reduced ACSL4 levels (Fig. 9E-G). These data collectively indicate that CKLF1 exerts its regulatory effects on myocardial ferroptosis via the Keap1/Nrf2/HO-1 signalling pathway.

## Discussion

Ferroptosis is an emerging form of regulated cell death characterized by distinct metabolic pathways involving iron homeostasis, amino acid metabolism, and lipid peroxidation[16]. This process is initiated when intracellular iron overload leads to excessive binding of  $\text{Fe}^{3+}$  to transferrin, followed by endosomal uptake via transferrin receptors[17–19]. Subsequent enzymatic reduction to  $\text{Fe}^{2+}$  facilitates cytoplasmic iron release, where it participates in Fenton reactions that generate reactive oxygen species (ROS)[7, 20]. These ROS drive extensive lipid peroxidation, ultimately triggering ferroptotic cell death[21]. The system  $\text{xc}^-$ -glutathione axis serves as a critical regulatory mechanism in ferroptosis. Through cysteine uptake, this system maintains intracellular GSH levels, which support GPX4 activity. GPX4 functions as a key antioxidant enzyme that reduces lipid peroxides, thereby protecting cells from ferroptotic death[22, 23]. Lipid metabolism plays a pivotal role in ferroptosis susceptibility, with the balance between monounsaturated fatty acid phospholipids (MUFA-PLs) and polyunsaturated fatty acid phospholipids (PUFA-PLs) being particularly crucial. Acyl-CoA synthetase family members, especially long-chain family members, have been identified as important mediators of this process. Notably, ACSL4 has been demonstrated to significantly promote ferroptosis across multiple cell lines, although its genetic ablation does not completely abolish ferroptotic death[24–26]. Recent studies have reported ferroptotic cell death in cardiomyocytes during myocardial infarction[27].

Our results demonstrate that myocardial infarction (MI) in rats leads to dysregulated expression of key ferroptosis-regulating proteins, such as GPX4 and ACSL4. Moreover, we detected abnormal levels of intracellular iron, ROS, MDA and GSH in the infarcted area. These findings provide compelling evidence for the involvement of ferroptosis in myocardial infarction. Our results also suggest that in myocardial infarction, ferroptosis can be induced through multiple pathways by affecting iron metabolism, amino

acid metabolism and lipid metabolism. Consistent with the in vivo observations, OGD-treated H9c2 cardiomyocytes exhibited similar alterations in GPX4 and ACSL4 expression. OGD-induced cardiomyocytes displayed significant abnormalities in total iron levels, ROS, MDA, and GSH levels, further corroborating the occurrence of ferroptosis in cardiomyocytes following myocardial infarction.

As a newly discovered chemokine, the main biological function of CKLF1 is chemotaxis, which plays a role in cellular inflammation and immune responses. A large number of studies have shown that CKLF1 is involved in various inflammatory diseases, such as extracellular matrix inflammation of chondrocytes[28], cellular inflammation in liver cancer[29], renal inflammation[30], inflammation in the ischaemic area of stroke[31], pulmonary inflammation in asthma[32], and vascular inflammation[33]. An earlier clinical study demonstrated that CKLF1 could improve cardiac function in patients after myocardial infarction[12]. Additionally, research has shown that CKLF1 can mobilize CD3 + cells in the peripheral blood of mice after myocardial infarction to enter the infarcted area and repair the damaged myocardium. Our previous studies have shown that in the rat AMI model, the expression of CKLF1 is increased in the infarct area, especially in the infarct border zone. In the H9c2 OGD model, the expression of CKLF1 is also increased. These preliminary results suggest that CKLF1 plays a role during myocardial infarction. Our current research further confirms that in the rat AMI model, the use of CKLF1-neutralizing antibodies can inhibit the expression of CKLF1 and thereby reduce the infarct area, as well as serum enzymes levels. After OGD in H9c2 cells, the CKLF1 antagonist peptide segment C19 also inhibited the expression of CKLF1 and alleviated cell damage. Therefore, CKLF1 represents a promising novel drug target for the treatment of myocardial infarction. Our experimental data, utilizing CKLF1-neutralizing antibodies and antagonistic peptides, further support the feasibility of developing therapeutics targeting CKLF1. Moreover, inhibition of CKLF1 expression can downregulate the expression of ferroptosis-related genes GPX4 and ACSL4. This further elucidates the molecular mechanism through which CKLF1 contributes to myocardial infarction, thereby providing stronger support for the development of targeted therapies.

During the inflammatory phase of myocardial infarction, chemokines promote the infiltration of leukocytes, which in turn secrete additional chemokines, establishing a positive feedback loop that amplifies the local inflammatory response[34]. In the repair phase, fibroblasts release chemokines to recruit additional leukocytes to the infarcted region. Upon recruitment, these leukocytes further secrete chemokines that exert paracrine effects on both fibroblasts and endothelial cells. This intricate chemokine-mediated crosstalk critically regulates neovascularization and extracellular matrix (ECM) synthesis, thereby playing a pivotal role in cardiac remodelling[35, 36]. Indeed, our previous investigations demonstrated that CKLF1 promotes microglial polarization and neutrophil recruitment, thereby exacerbating cerebral ischaemia-reperfusion injury in a murine stroke model[5, 37]. Therefore, the interaction between CKLF1 and leukocytes represents a crucial mechanism through which CKLF1 exerts its biological functions. Our previous findings demonstrated that CKLF1 colocalizes with neutrophils and macrophages in the infarcted myocardium of rats. These observations provide preliminary evidence for CKLF1-mediated communication with leukocytes following myocardial

infarction. However, the underlying mechanisms of this interaction and its potential association with ferroptosis remain to be fully elucidated.

Nrf2 is a crucial antioxidant transcription factor that plays a central role in the p62-Keap1-Nrf2 pathway. Nrf2 can bind to AREs in the promoter and activate various antioxidant enzymes to combat oxidative stress. HO-1 is the main protein that regulates ROS responses, which occur downstream of Nrf2. The Nrf2 signalling pathway also plays a core role in sorafenib resistance and ferroptosis induction through HO-1[14, 38]. Studies have shown that many proteins in the Nrf2 signalling pathway, such as Keap1, can be activated by lipid peroxidation and participate in ferroptosis[39, 40]. Our research revealed that the Keap1/Nrf2/HO-1 signalling pathway was activated after myocardial infarction in rats. Nrf2 can target iron transport proteins and glutathione peroxidase 4 in cells to regulate ferroptosis [41–43]. In the early and middle stages of MI, the activity of Hmox1 is enhanced through the Nrf2/Hmox1 pathway, leading to iron overload and resulting in ferroptosis of myocardial cells[44]. Our research revealed that Keap1/Nrf2/HO-1 signalling pathway is activated in H9c2 cardiomyocytes after OGD and affect the expression of ferroptosis-related proteins. When Nrf2 inhibitors are used, Nrf2 signalling pathway and the expression of ferroptosis-related proteins are reversed. These results indicate that CKLF1 can regulate ferroptosis in cardiomyocytes through the Keap1/Nrf2/HO-1 signalling pathway.

Our study has several limitations. First, the direct molecular mechanism through which CKLF1 interacts with the Keap1, Nrf2, and HO-1 signalling pathways remains unclear, and requires further investigation using protein spectrometry and other techniques. Second, our study focused on ferroptosis in myocardial cells during myocardial infarction. Whether CKLF1 can affect immune cells in the infarct area and indirectly affect myocardial cells, thereby affecting the ferroptotic microenvironment, is an interesting question worthy of further investigation. In fact, our previous research revealed that, CKLF1 colocalizes with macrophages and neutrophils in the infarct area, which will be the direction of our next study. The results of this study provide support for the development of protein drug and gene therapy strategies based on CKLF1.

## Conclusion

In summary, this study demonstrated that CKLF1 is involved in the ferroptosis of cardiomyocytes in myocardial infarction, mainly through the Keap1/Nrf2/HO-1 signalling pathway. This study clarified the role of CKLF1 in myocardial infarction and explored its specific molecular mechanism, providing a new direction for the study of disease pathogenesis and a new drug target for disease treatment.

## Declarations

## Declaration of competing interest

The authors declare that they have no conflicts of interest.

# Authorship and contributorship

Juling Feng: conceptualization and, writing - original draft; Lei Zhao: data curation and formal analysis; Haodong Chen: data curation and methodology; Qidi Ai, PhD: conceptualization, data curation, methodology, validation, and writing - review and editing; Shifeng Chu, PhD, and Yantao Yang, Prof. Dr: conceptualization, validation, and writing - review and editing.

## Declarations

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## Clinical trial number

not applicable.

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## Author Contribution

Juling Feng: conceptualization and, writing - original draft; Lei Zhao: data curation and formal analysis; Haodong Chen: data curation and methodology; Qidi Ai, PhD: conceptualization, data curation, methodology, validation, and writing - review and editing; Shifeng Chu, PhD, and Yantao Yang, Prof. Dr: conceptualization, validation, and writing - review and editing.

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## Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

## Statement of ethics

All operations were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were authorized by the Peking Union Medical College and the Chinese Academy of Medical Sciences Animal Care Committee (number: 00007889).

## Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use generative AI or AI-assisted technologies in the development of this manuscript.

## References

1. Roth, G. A., Mensah, G. A. & Fuster, V. The Global Burden of Cardiovascular Diseases and Risks: A Compass for Global Action. *J. Am. Coll. Cardiol.* **76** (25), 2980–2981 (2020).
2. Liu, Y., Xu, J., Wu, M., Kang, L. & Xu, B. The effector cells and cellular mediators of immune system involved in cardiac inflammation and fibrosis after myocardial infarction. *J. Cell. Physiol.* **235** (12), 8996–9004 (2020).
3. Liu, D. D. et al. Progress in pharmacological research of chemokine like factor 1 (CKLF1). *Cytokine* **102**, 41–50 (2018).
4. Yang, G. Y., Chen, X., Sun, Y. C., Ma, C. L. & Qian, G. Chemokine-like factor 1 (CLFK1) is over-expressed in patients with atopic dermatitis. *Int. J. Biol. Sci.* **9** (8), 759–765 (2013).
5. Chen, C. et al. CKLF1 Aggravates Focal Cerebral Ischemia Injury at Early Stage Partly by Modulating Microglia/Macrophage Toward M1 Polarization Through CCR4. *Cell. Mol. Neurobiol.* **39** (5), 651–669 (2019).
6. Feng, J. et al. Chemokine-like factor 1 (CKLF1) is expressed in myocardial ischemia injury *in vivo* and *in vitro*. *Biocell* **48** (6), 981–990 (2024).
7. Dixon, S. J. et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* **149** (5), 1060–1072 (2012).
8. Ma, H., Dong, Y., Chu, Y., Guo, Y. & Li, L. The mechanisms of ferroptosis and its role in alzheimer's disease. *Front. Mol. Biosci.* **9**, 965064 (2022).
9. Chen, X., Kang, R., Kroemer, G. & Tang, D. Broadening horizons: the role of ferroptosis in cancer. *Nat. Rev. Clin. Oncol.* **18** (5), 280–296 (2021).
10. Li, Y. et al. Ischemia-induced ACSL4 activation contributes to ferroptosis-mediated tissue injury in intestinal ischemia/reperfusion. *Cell. Death Differ.* **26** (11), 2284–2299 (2019).
11. Li, W. et al. Ferroptotic cell death and TLR4/Trif signaling initiate neutrophil recruitment after heart transplantation. *J. Clin. Invest.* **129** (6), 2293–2304 (2019).
12. Yang, B. et al. [Effects of *in vivo* transfer of human chemokine-like factor 1 gene on cardiac function after acute myocardial infarction in rats]. *Beijing Da Xue Xue Bao Yi Xue Ban.* **41** (2), 144–147 (2009).

13. Park, T. J. et al. Quantitative proteomic analyses reveal that GPX4 downregulation during myocardial infarction contributes to ferroptosis in cardiomyocytes. *Cell. Death Dis.* **10** (11), 835 (2019).
14. Chen, Q. M. Nrf2 for protection against oxidant generation and mitochondrial damage in cardiac injury. *Free Radic Biol. Med.* **179**, 133–143 (2022).
15. Deng, X., Chu, W., Zhang, H. & Peng, Y. Nrf2 and Ferroptosis: A New Research Direction for Ischemic Stroke. *Cell. Mol. Neurobiol.* **43** (8), 3885–3896 (2023).
16. Sharma, A. & Flora, S. J. S. Positive and Negative Regulation of Ferroptosis and Its Role in Maintaining Metabolic and Redox Homeostasis. *Oxid. Med. Cell. Longev.* **2021**, 9074206 (2021).
17. Koleini, N., Shapiro, J. S., Geier, J. & Ardehali, H. Ironing out mechanisms of iron homeostasis and disorders of iron deficiency. *J. Clin. Invest.* **131**(11) (2021).
18. Wallace, D. F. The Regulation of Iron Absorption and Homeostasis. *Clin. Biochem. Rev.* **37** (2), 51–62 (2016).
19. Galy, B., Conrad, M. & Muckenthaler, M. Mechanisms controlling cellular and systemic iron homeostasis. *Nat. Rev. Mol. Cell. Biol.* **25** (2), 133–155 (2024).
20. Chen, X., Yu, C., Kang, R. & Tang, D. Iron Metabolism in Ferroptosis. *Front. Cell. Dev. Biol.* **8**, 590226 (2020).
21. Yu, Y. et al. The ferroptosis inducer erastin enhances sensitivity of acute myeloid leukemia cells to chemotherapeutic agents. *Mol. Cell. Oncol.* **2** (4), e1054549 (2015).
22. Zhang, W., Liu, Y., Liao, Y., Zhu, C. & Zou, Z. GPX4, ferroptosis, and diseases. *Biomed. Pharmacother.* **174**, 116512 (2024).
23. Cui, C., Yang, F. & Li, Q. Post-Translational Modification of GPX4 is a Promising Target for Treating Ferroptosis-Related Diseases. *Front. Mol. Biosci.* **9**, 901565 (2022).
24. Zou, Y. et al. Cytochrome P450 oxidoreductase contributes to phospholipid peroxidation in ferroptosis. *Nat. Chem. Biol.* **16** (3), 302–309 (2020).
25. Doll, S. et al. ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. *Nat. Chem. Biol.* **13** (1), 91–98 (2017).
26. Ding, K. et al. Acyl-CoA synthase ACSL4: an essential target in ferroptosis and fatty acid metabolism. *Chin. Med. J. (Engl.)*. **136** (21), 2521–2537 (2023).
27. Wu, X., Li, J., Cheng, H. & Wang, L. Ferroptosis and Lipid Metabolism in Acute Myocardial Infarction. *Rev. Cardiovasc. Med.* **25** (5), 149 (2024).
28. Wang, H., Wu, Z. & Xu, K. CKLF1 interference alleviates IL–1 $\beta$ –induced inflammation, apoptosis and degradation of the extracellular matrix in chondrocytes via CCR5. *Exp. Ther. Med.* **25** (6), 303 (2023).
29. Liu, Y. et al. CKLF1 Enhances Inflammation-Mediated Carcinogenesis and Prevents Doxorubicin-Induced Apoptosis via IL6/STAT3 Signaling in HCC. *Clin. Cancer Res.* **25** (13), 4141–4154 (2019).
30. Chen, C., Ai, Q. & Wei, Y. Hydroxytyrosol protects against cisplatin-induced nephrotoxicity via attenuating CKLF1 mediated inflammation, and inhibiting oxidative stress and apoptosis. *Int. Immunopharmacol.* **96**, 107805 (2021).

31. Ai, Q. et al. IMM-H004 Protects against Cerebral Ischemia Injury and Cardiopulmonary Complications via CKLF1 Mediated Inflammation Pathway in Adult and Aged Rats. *Int. J. Mol. Sci.* **20**(7) (2019).
32. Tian, L. et al. The CKLF1-C19 peptide attenuates allergic lung inflammation by inhibiting CCR3- and CCR4-mediated chemotaxis in a mouse model of asthma. *Allergy* **66** (2), 287–297 (2011).
33. Zhang, T. et al. Effects of chemokine-like factor 1 on vascular smooth muscle cell migration and proliferation in vascular inflammation. *Atherosclerosis* **226** (1), 49–57 (2013).
34. Hilgendorf, I., Frantz, S. & Frangogiannis, N. G. Repair of the Infarcted Heart: Cellular Effectors, Molecular Mechanisms and Therapeutic Opportunities. *Circ. Res.* **134** (12), 1718–1751 (2024).
35. DeLeon-Pennell, K. Y., Meschiari, C. A., Jung, M. & Lindsey, M. L. Matrix Metalloproteinases in Myocardial Infarction and Heart Failure. *Prog Mol. Biol. Transl Sci.* **147**, 75–100 (2017).
36. Lindsey, M. L. et al. Cellular Interactions of Cardiac Repair After Myocardial Infarction. *Cells* **14**(23) (2025).
37. Chen, C., Chu, S. F., Ai, Q. D., Zhang, Z. & Chen, N. H. CKLF1/CCR5 axis is involved in neutrophils migration of rats with transient cerebral ischemia. *Int. Immunopharmacol.* **85**, 106577 (2020).
38. Lu, J., Zhao, Y., Liu, M., Lu, J. & Guan, S. Toward improved human health: Nrf2 plays a critical role in regulating ferroptosis. *Food Funct.* **12** (20), 9583–9606 (2021).
39. Dinkova-Kostova, A. T., Kostov, R. V. & Canning, P. Keap1, the cysteine-based mammalian intracellular sensor for electrophiles and oxidants. *Arch. Biochem. Biophys.* **617**, 84–93 (2017).
40. Levonen, A. L. et al. Cellular mechanisms of redox cell signalling: role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products. *Biochem. J.* **378** (Pt 2), 373–382 (2004).
41. Harada, N. et al. Nrf2 regulates ferroportin 1-mediated iron efflux and counteracts lipopolysaccharide-induced ferroportin 1 mRNA suppression in macrophages. *Arch. Biochem. Biophys.* **508** (1), 101–109 (2011).
42. Osburn, W. O. et al. Nrf2 regulates an adaptive response protecting against oxidative damage following diquat-mediated formation of superoxide anion. *Arch. Biochem. Biophys.* **454** (1), 7–15 (2006).
43. Salazar, M., Rojo, A. I., Velasco, D., de Sagarra, R. M. & Cuadrado, A. Glycogen synthase kinase-3 $\beta$  inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2. *J. Biol. Chem.* **281** (21), 14841–14851 (2006).
44. Dodson, M., Castro-Portuguez, R. & Zhang, D. D. NRF2 plays a critical role in mitigating lipid peroxidation and ferroptosis. *Redox Biol.* **23**, 101107 (2019).

## Figures

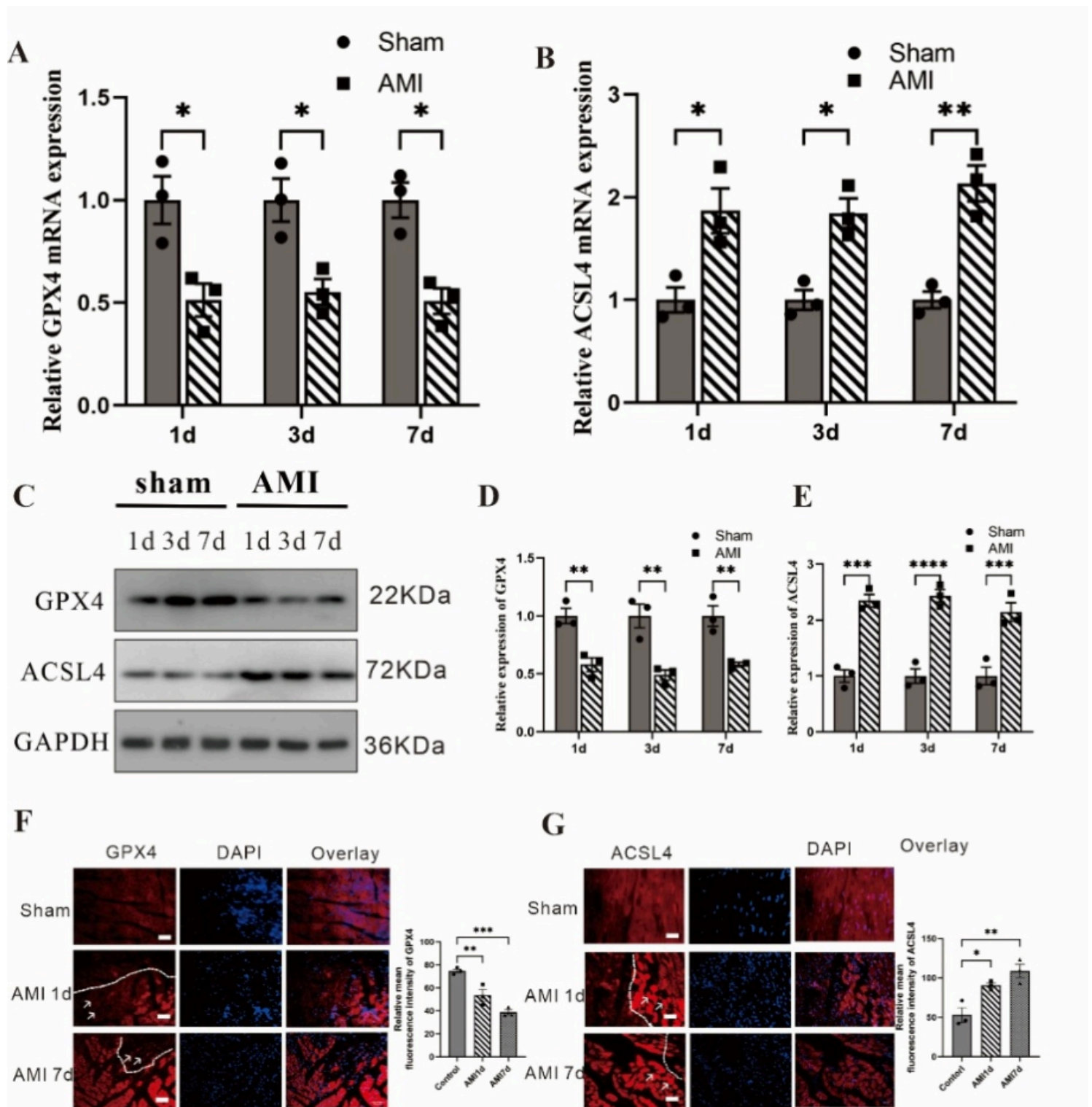


**Figure 1**

The presence of ferroptosis in the infarcted area of the rat myocardium. (A,B) mRNA expression levels of GPX4 and ACSL4 in the infarct area 1, 3, and 7 days after AMI in rats. (C-E) GPX4 and ACSL4 protein expression and quantitative analysis in the infarct area 1/3/7 days after AMI in rats. (F,G) Immunofluorescence staining of GPX4(red) and ACSL4(red) in the infarct area 1, 3, and 7 days after AMI

in rats. Scale bar = 50μm. Arrows points to the infarct border zone. Values are presented as Mean±SEM. n= 3. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$ .

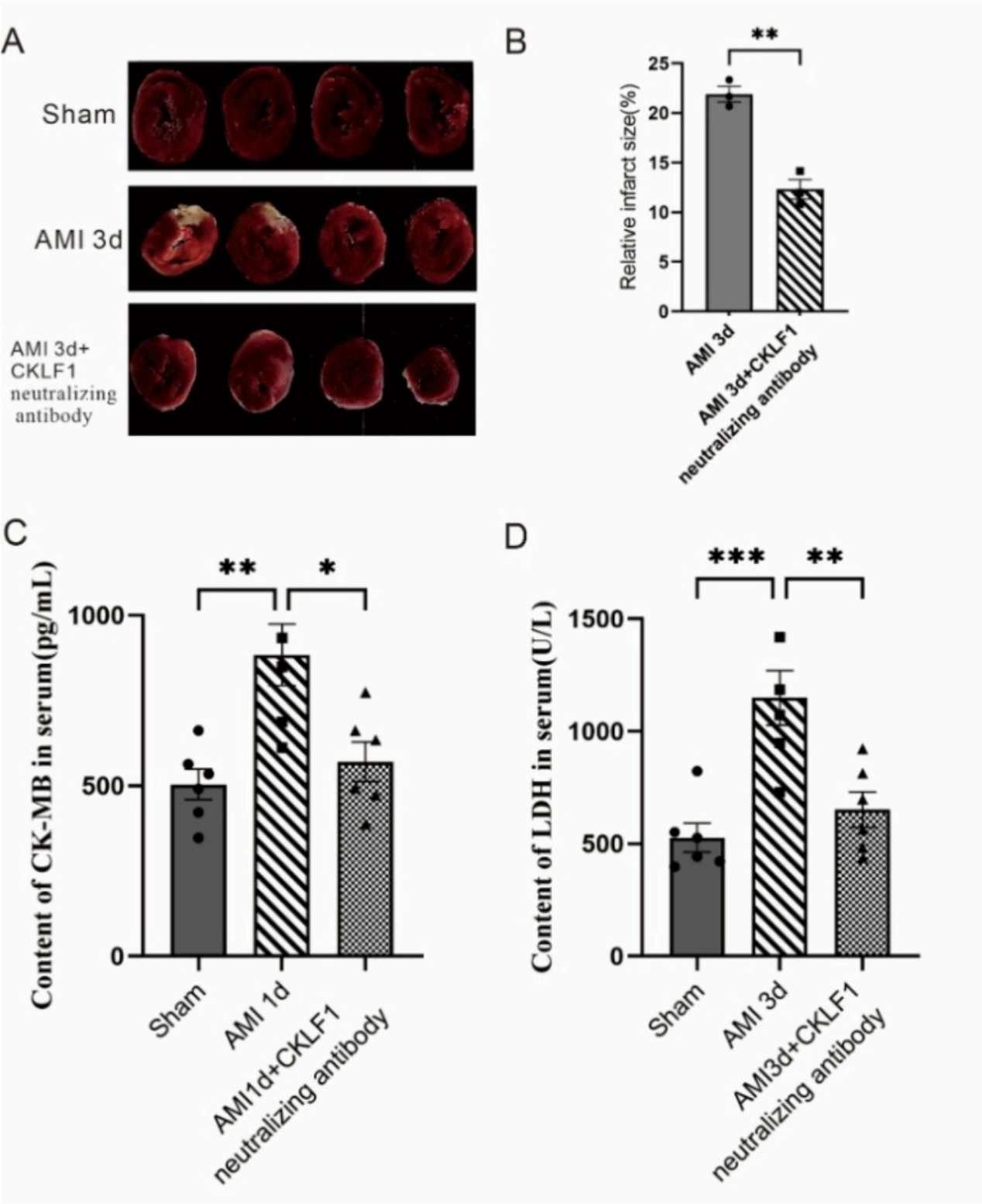


Figure 2

CKLF1 prevents myocardial infarction in rats. (A.B) TTC staining was used to detect the area of myocardial infarction in sham, AMI and AMI+CKLF1 neutralizing antibody groups in rats. n=3. (C.D) CK-

MB and LDH content in rat serum.  $n=6$ . Values are presented as Mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .

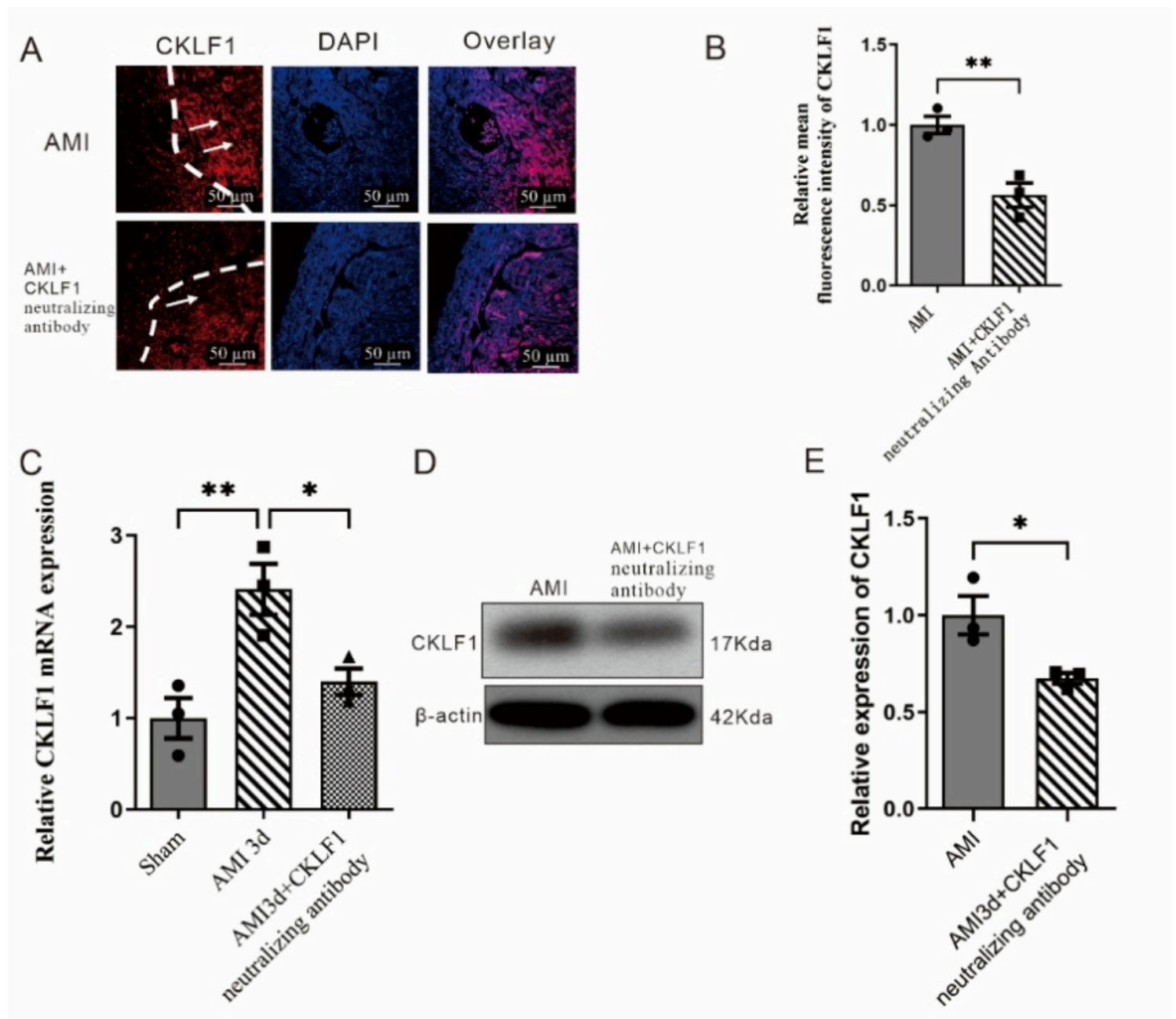


**Figure 3**

CKLF1 neutralizing antibody reduces CKLF1 expression in the infarcted area of rats. (A,B) immunofluorescence staining and quantitative analysis of CKLF1(Red) in the infarcted area of AMI rats. Scale bar = 50 $\mu$ m. CKLF1 neutralizing antibody =40 $\mu$ L/ rat. (C) qPCR was used to detect the mRNA expression of CKLF1 in the infarcted area of AMI rats. (D,E) expression and quantitative analysis of CKLF1 protein in infarcted area of sham, AMI and AMI+CKLF1 neutralizing antibody groups in rats. Values are presented as Mean $\pm$ SEM.  $n=3$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ .

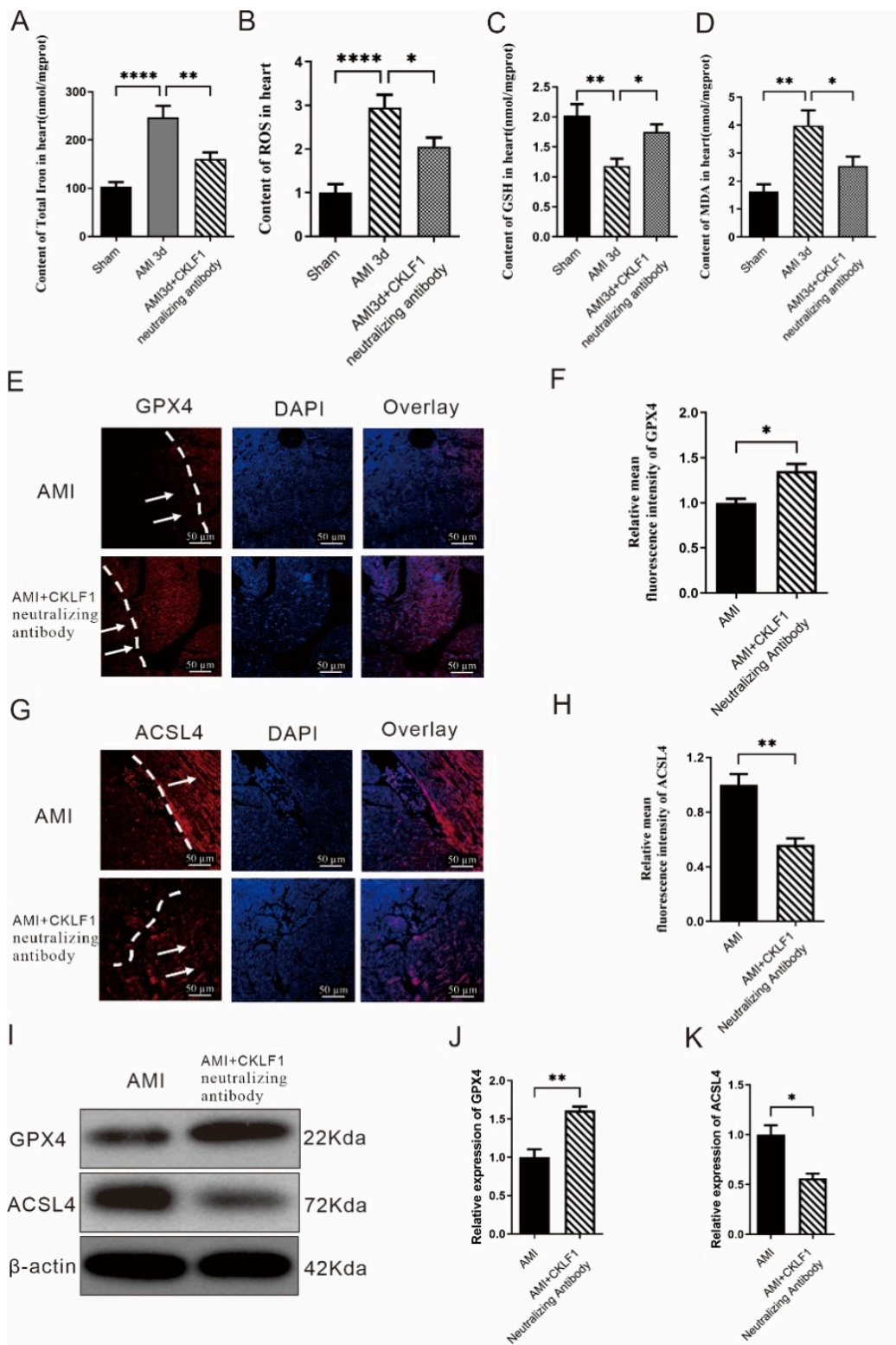


**Figure 4**

CKLF1 neutralizing antibody reduces CKLF1 expression in the infarcted area of rats. (A,B) immunofluorescence staining and quantitative analysis of CKLF1(Red) in the infarcted area of AMI rats. Scale bar = 50 $\mu$ m. CKLF1 neutralizing antibody = 40 $\mu$ L/ rat. (C) qPCR was used to detect the mRNA expression of CKLF1 in the infarcted area of AMI rats. (D,E) expression and quantitative analysis of

CKLF1 protein in infarcted area of sham, AMI and AMI+CKLF1 neutralizing antibody groups in rats. Values are presented as Mean $\pm$ SEM. n=3. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

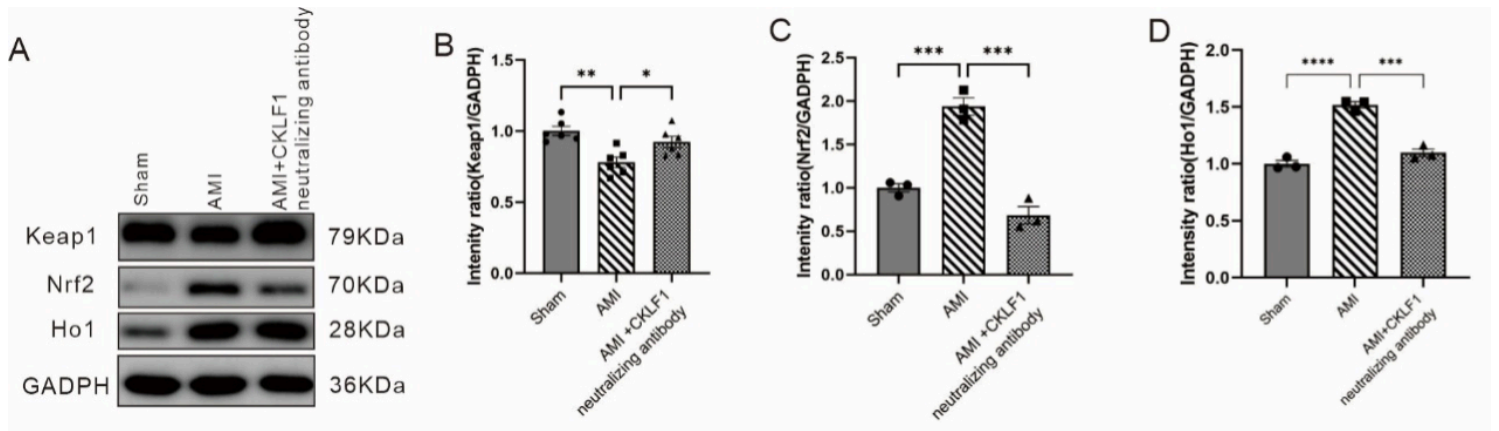


**Figure 5**

The expression of Keap1/Nrf2/Ho1 proteins in the infarcted area after AMI in rats. (A-D) Protein expression and quantitative investigation of Keap1, Nrf2 and Ho1 in sham,AMI and AMI+CKLF1 neutralizing antibody groups in rats. Values are presented as Mean  $\pm$  SEM. n=3. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

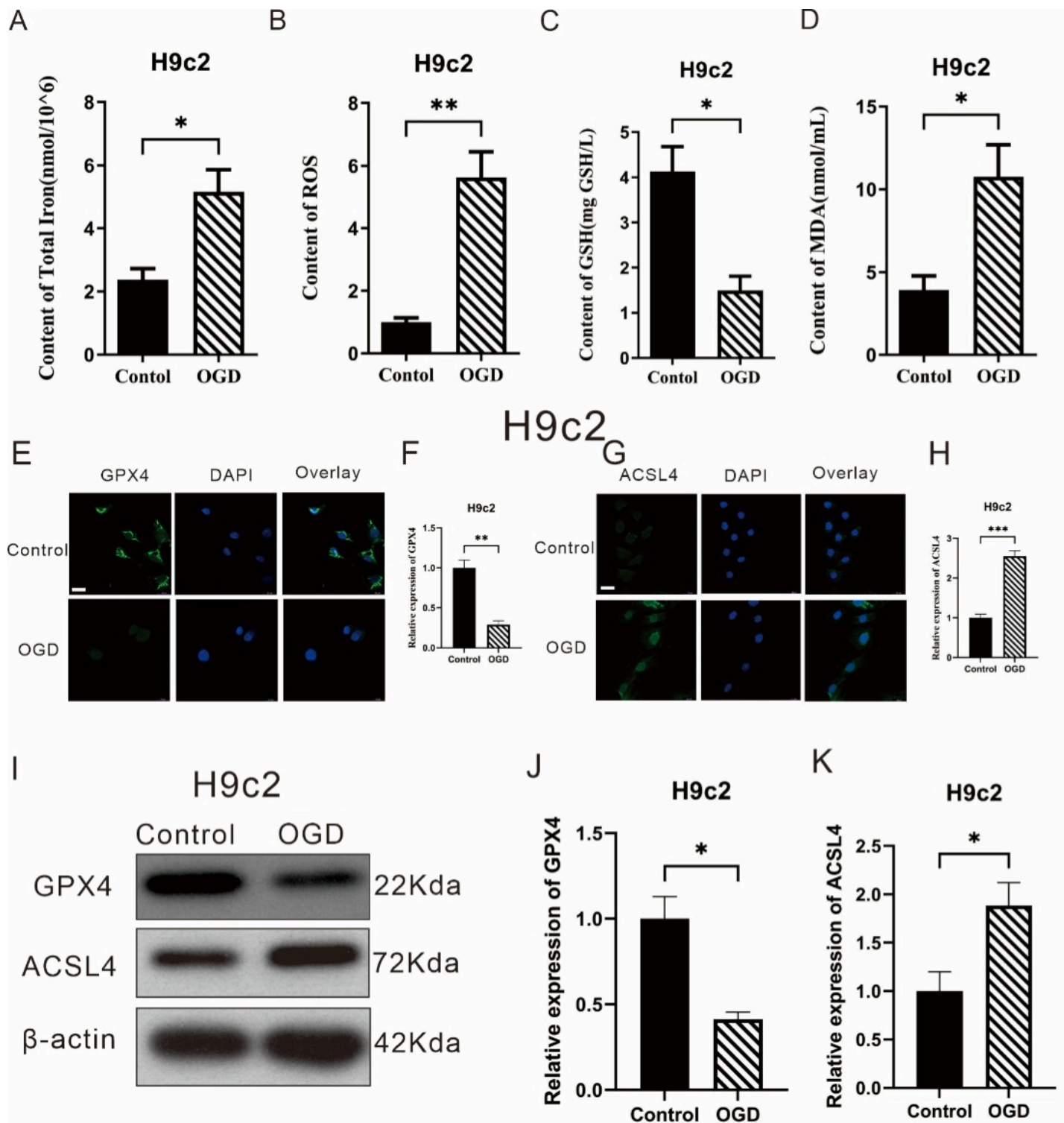


**Figure 6**

The expression of Keap1/Nrf2/Ho1 proteins in the infarcted area after AMI in rats. (A-D) Protein expression and quantitative investigation of Keap1, Nrf2 and Ho1 in sham,AMI and AMI+CKLF1 neutralizing antibody groups in rats. Values are presented as Mean  $\pm$  SEM. n=3. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$ .

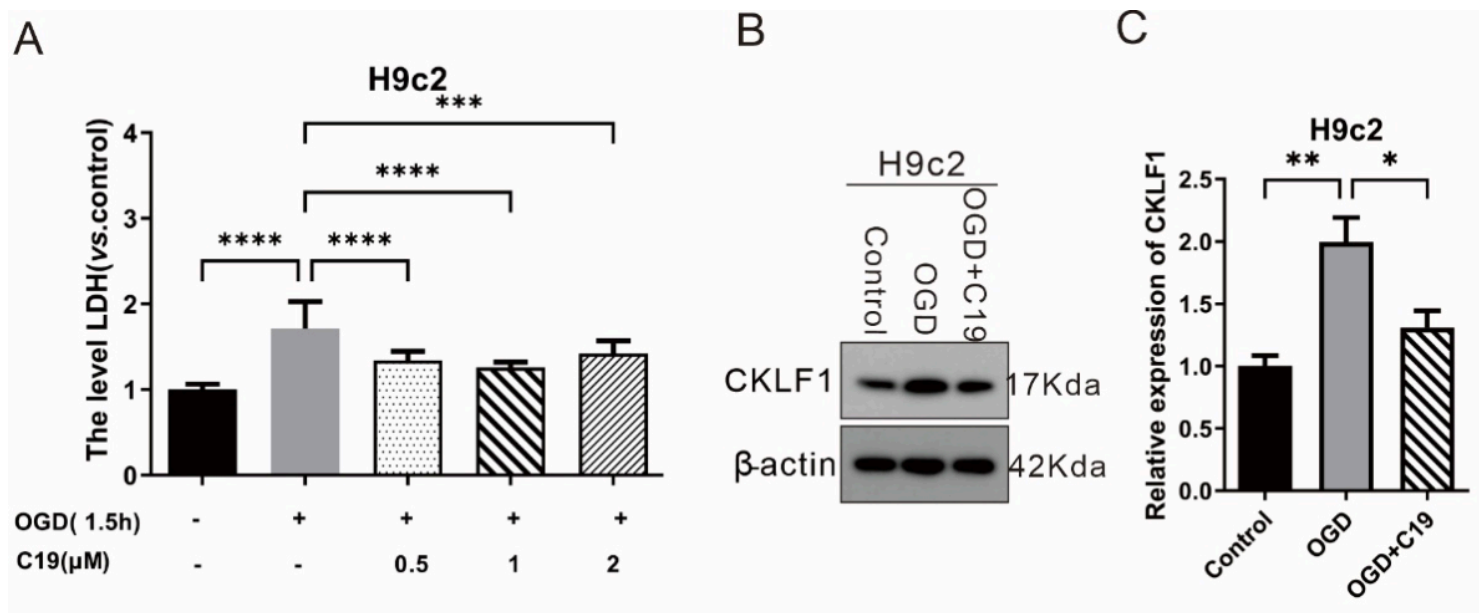


**Figure 7**

C19 alleviates cardiomyocyte injury and reduces CKLF1 expression after OGD. (A) LDH content in H9c2 cell supernatant at different times after OGD and C19 treatment. The experiment was repeated six times. (B.C) Expression and quantitative analysis of CKLF1 proteins in H9c2 cells after adding C19 and OGD for 12h. Values are expressed as Mean±SEM. The experiment was repeated three times. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .

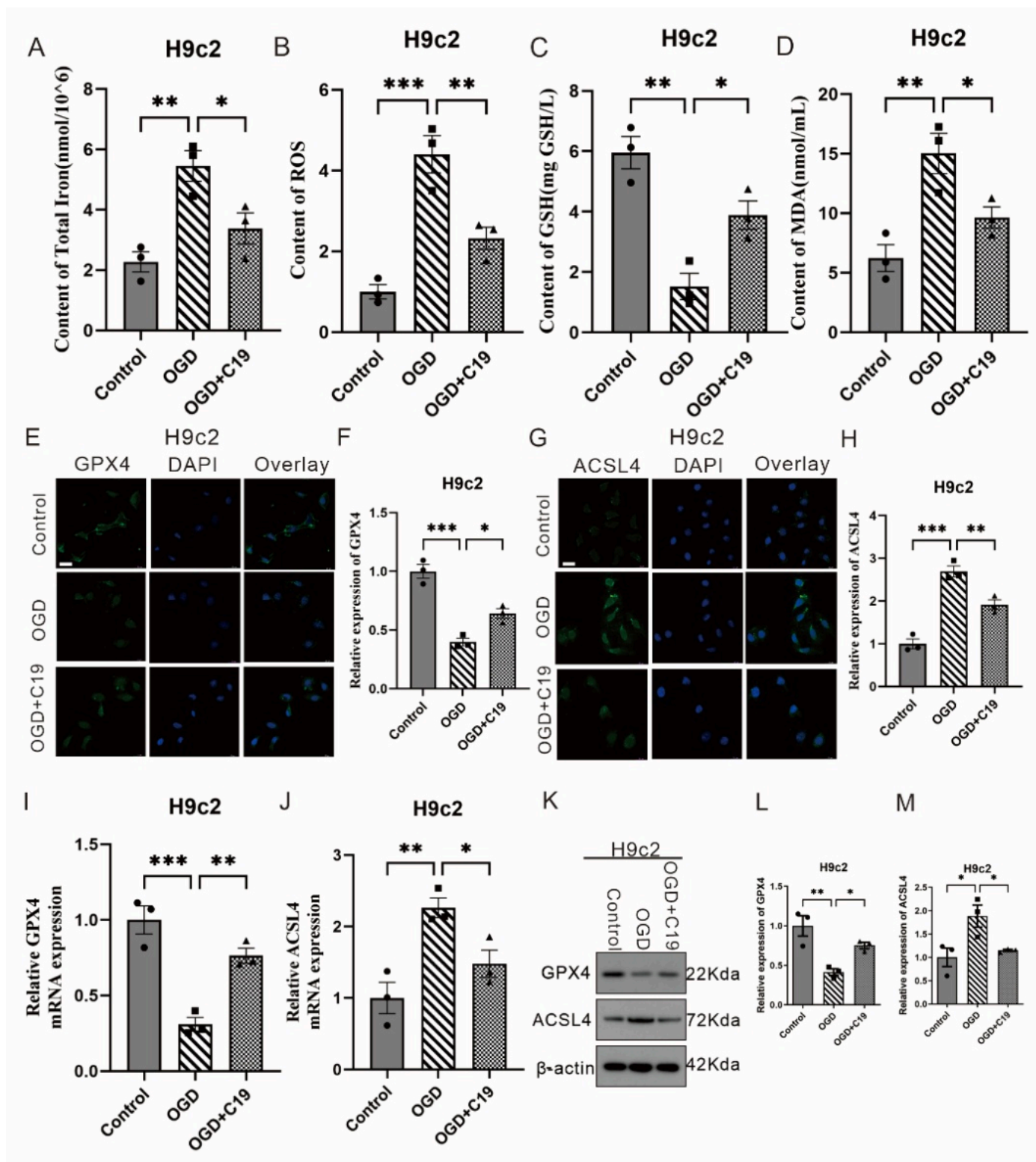


**Figure 8**

C19 inhibits OGD-induced ferroptosis in cardiomyocytes. (A-D) Contents of iron, ROS, GSH and MDA in H9c2 cardiomyocytes treated with C19 and OGD for 12h. (E-H) Immunofluorescence staining and quantitative analysis of GPX4(green) and ACSL4(green) in H9c2 cells. Scale = 25 $\mu$ m. (I-J) The mRNA expression levels of GPX4 and ACSL4 in H9c2 cells. (K-M) Expression and quantitative analysis of GPX4

and ACSL4 proteins in H9c2 cells. Values are expressed as Mean±SEM. The experiment was repeated three times. \*  $p < 0.05$  \*\*  $p < 0.01$  \*\*\*  $p < 0.001$

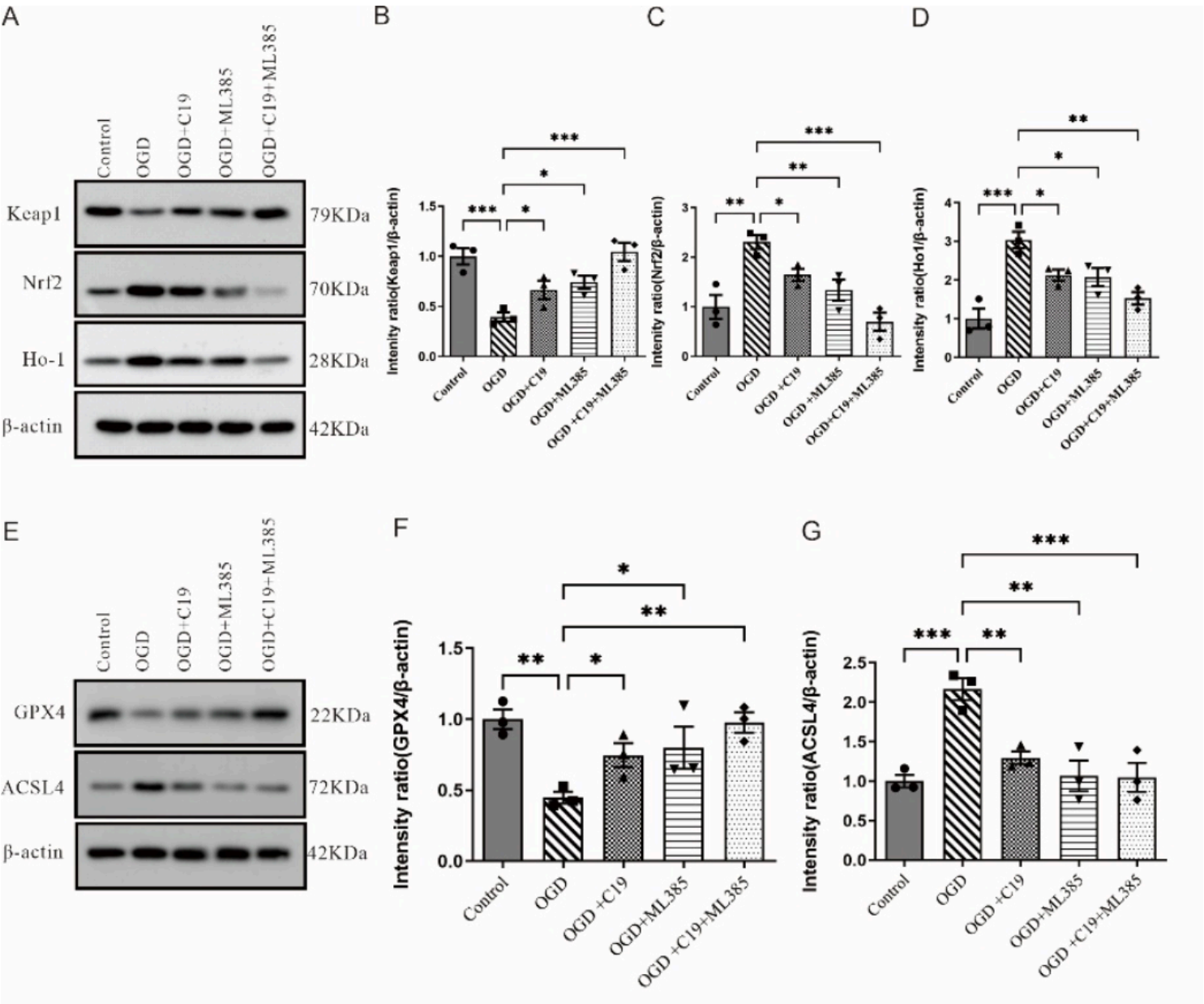


**Figure 9**

CKLF1 regulates ferroptosis in cardiomyocytes through the Keap1/Nrf2/Ho1 signaling pathway. (A-G) Expression and quantitative analysis of Keap1, Nrf2 and HO-1 proteins in cardiomyocytes treated with OGD, C19 and ML385. The values are expressed as Mean±SEM. The experiment was repeated three times. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## Supplementary Files

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