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Ferroptotic cell death and TLR4/Trif signaling initiate neutrophil recruitment after heart transplantation

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Abstract

Non-apoptotic forms of cell death can trigger sterile inflammation through the release of danger-associated molecular patterns, which are recognized by innate immune receptors. However, despite years of investigation the mechanisms which initiate inflammatory responses after heart transplantation remain elusive. Here, we demonstrate that ferrostatin-1 (Fer-1), a specific inhibitor of ferroptosis, decreases the level of pro-ferroptotic hydroperoxy-arachidonyl-phosphatidylethanolamine, reduces cardiomyocyte cell death and blocks neutrophil recruitment following heart transplantation. Inhibition of necroptosis had no effect on neutrophil trafficking in cardiac grafts. We extend these observations to a model of coronary artery ligation-induced myocardial ischemia reperfusion injury where inhibition of ferroptosis resulted in reduced infarct size, improved left ventricular systolic function, and reduced left ventricular remodeling. Using intravital imaging of cardiac transplants, we uncover that ferroptosis orchestrates neutrophil recruitment to injured myocardium by promoting adhesion of neutrophils to coronary vascular endothelial cells through a TLR4/Trif/type I IFN signaling pathway. Thus, we have discovered that inflammatory responses after cardiac transplantation are initiated through ferroptotic cell death and TLR4/Trif-dependent signaling in graft endothelial cells. These findings provide a platform for the development of therapeutic strategies for heart transplant recipients and patients, who are vulnerable to ischemia reperfusion injury following restoration of coronary blood flow.
Introduction

Ischemia reperfusion injury (IRI) following heart transplantation is a clinically significant form of sterile inflammation. IRI-mediated primary graft dysfunction complicates the post-operative course of up to 28% of human cardiac transplant recipients (1). Of note, the development of primary graft dysfunction can result in an early mortality rate of approximately 10% and is also associated with significantly lower survival rates at one year after transplantation (2). While several donor and recipient factors have been identified as risk factors for the development of primary graft dysfunction after heart transplantation, the cellular and molecular mechanisms by which IRI induces myocardial inflammation are not well understood.

Neutrophils are recruited to sites of sterile inflammation. For example, our group and others have reported that neutrophils are critical mediators of primary graft dysfunction after lung transplantation (3). Similarly, neutrophils are also recruited to injured myocardium in humans and experimental animals, where they cause tissue damage (4). It is the prevailing notion that neutrophil recruitment during sterile injury is triggered through the release of damage-associated molecular patterns (DAMPs) from dead cells and subsequent engagement of innate immune receptors such as toll like receptors (TLRs) (5). TLR activation initiates signaling via the adaptor proteins MyD88 or Trif. Our current understanding is that following ischemia reperfusion injury, cells die through a combination of apoptosis and non-apoptotic forms of programmed cell death (6). It is further postulated that non-apoptotic forms of programmed cell death such as necroptosis or ferroptosis result in the release of DAMPs that ultimately result in the initiation of inflammation, referred to as necroinflammation (7). The precise cell death
pathways and signaling events that orchestrate early inflammation after heart transplantation are unknown.

The cellular and molecular requirements that regulate specific steps of neutrophil trafficking differ between various tissues and organs (8). Our laboratory has developed methods to image beating murine hearts by intravital two-photon microscopy, which allows for a detailed analysis of leukocyte trafficking in cardiac grafts in real time (9). Using this approach, we have recently described that neutrophil extravasation into reperfused transplanted hearts is mediated by tissue-resident CCR2+ macrophages through a TLR9/MyD88-dependent pathway (10). Here, we took advantage of intravital imaging to examine the pathways that regulate the initial recruitment of neutrophils to cardiac grafts. We have discovered that the early cues allowing neutrophils to slow down in the vasculature and to adhere to the vessel wall during IRI are mediated through ferroptotic cell death and TLR4/Trif-dependent signaling in graft endothelial cells.
Results

Ferroptosis regulates cell death and neutrophil recruitment after heart transplantation

To interrogate the initial recruitment of neutrophils by intravital two-photon imaging following cardiac transplantation we engrafted B6 wildtype hearts into syngeneic B6 LysM-GFP neutrophil reporter mice (9, 10). Consistent with our previous reports, neutrophils are rapidly recruited to heart grafts immediately following reperfusion (Figure 1A, Supplemental Video 1). Only few cardiomyocytes stained for cleaved caspase-3 in these grafts and we did not observe differences in neutrophil recruitment kinetics after treating recipient mice with the pan-caspase inhibitor Z-VAD-FMK (Supplemental Figure 1, Supplemental Video 2). These findings indicate that classical apoptotic pathways of cell death in the cardiac graft do not play an important role in the regulation of early neutrophil recruitment following reperfusion. Treatment of heart recipients with the RIPK1 inhibitor necrostatin-1 (Nec-1) at the time of transplantation, however, resulted in significant increases in rolling velocities. Very few neutrophils slowed down sufficiently to transition to adherence to the walls of the coronary veins (Fig. 1 B, E; Supplemental Video 3). Consequently, we observed significant reductions in their density and also an impairment in their extravasation (Fig. 1 F, G). Nec-1 has been frequently used to block necroptosis in various experimental systems (11). To test the role of necroptosis genetically, we next transplanted hearts that lack expression of RIPK3, a kinase that is essential in triggering necroptosis (12). Transplantation of RIPK3-deficient hearts mirrored neutrophil behavior observed after engraftment of wildtype hearts (Fig. 1C, E-G; Supplemental Video 4). As reports exist that Nec-1 can inhibit both necroptosis and
ferroptosis we next interrogated the ferroptotic pathway of cell death (13). Treatment of heart recipients with ferrostatin (Fer-1), a selective inhibitor of ferroptosis (14), yielded results similar to those observed after administration of Nec-1 (Fig. 1D-G; Supplemental Video 5). Of note, inhibition of ferroptosis did not impact the rate at which neutrophils were recruited to the graft and Fer-1 treatment did not directly inhibit neutrophil chemotaxis (Fig. 1H; Supplemental Fig. 2). Consistent with published reports of cell death in the setting of myocardial IRI, intravascular injection of ethidium homodimer 2 hours after transplantation revealed uptake of dye into clusters of cardiomyocytes located within the left ventricular (LV) myocardium (Fig. 1I). Administration of Fer-1 to recipients at the time of transplantation resulted in marked reductions in ethidium homodimer in cardiomyocytes compared to controls. Flow cytometric evaluation revealed that Fer-1 also inhibits the death of fibroblasts, but not endothelial cells, in heart grafts during IRI (Fig. 1J; Supplemental Fig. 3). In addition, through oxidative lipidomics we determined that a specific lipid peroxidation product, hydroperoxy-arachidonoylphosphatidylethanolamine (HOO-C20:4/C18:0-PE) that acts as a ferroptotic death signal, was reduced in whole tissue homogenates obtained from cardiac grafts after treatment with Fer-1(15) (Fig. 1K).

**Ferroptosis mediates cell death and tissue injury after non-transplant-related myocardial IRI**

To examine whether ferroptosis is responsible for cardiomyocyte cell death following IRI in the absence of exogenous immune cell recruitment, we employed Langendorff preparations. We observed that hearts subjected to 1 hour of ischemia
displayed a marked increase in total creatinine kinase (CK) activity released over a 30-minute reperfusion period compared to control hearts that were not subjected to ischemia. Hearts treated with Fer-1 demonstrated reduced levels of released total CK activity compared to vehicle-treated controls following IRI (Fig. 2A). Serial measurements of released CK activity revealed that Fer-1 decreased CK release after 15 minutes of reperfusion suggesting that ferroptosis may be selectively impacting cardiomyocyte cell death that occurs as a result of IRI rather than ischemia alone (Fig. 2B). Arachidonic acid containing phospholipids are key mediators of ferroptosis and oxidized arachidonic acid metabolites are associated with ferroptotic cell death (15). Measurement of arachidonic acid metabolites by mass spectrometry (LC-MS/MS) revealed increased abundance of several hydroxyeicosatetraenoic acid (HETE) and epoxyeicosatrienoic acids (EET) species as well as prostaglandin D2 in hearts subjected to IRI. Fer-1 treatment resulted in substantial reductions in the abundance of each of these lipid species. Among oxidized arachidonic acid metabolites increased following IRI, Fer-1 reduced the abundance of several species previously implicated as possible markers of ferroptosis including 5-HETE, 11-HETE, 12-HETE and 15-HETE (13, 16) (Fig. 2C). Fer-1 treatment also reduced the abundance of docosanoids and oxidized linoleic acid species consistent with the described mechanism of action of Fer-1 as a scavenger of peroxidated lipids (17) (Supplemental Fig. 4).

To delineate whether ferroptosis also regulates cardiomyocyte cell death in the context of myocardial infarction, we employed an in vivo model of closed-chest myocardial IRI (18). Administration of Fer-1 immediately following 90 minutes of IRI resulted in significant reductions in initial infarct area and neutrophil infiltration into the
heart 48 hours following myocardial infarction providing further evidence that ferroptosis is a critical mediator of early cardiomyocyte cell death and neutrophil recruitment in vivo (Fig. 2D-E). Echocardiographic analysis performed 4 weeks after myocardial infarction demonstrated improvements in left ventricular (LV) ejection fraction, reductions in LV systolic and diastolic chamber dimensions, and smaller akinetic areas in mice treated with Fer-1 (Fig. 2F-I). Pathological analysis 4 weeks after myocardial infarction revealed a smaller infarct size and reduced interstitial fibrosis in Fer-1-treated mice (Fig. 2J; Supplemental Fig. 5).

**Early steps of neutrophil recruitment after heart transplantation are mediated through a TLR4/Trif-dependent pathway**

Ferroptosis is an inflammatory form of regulated cell death that results in loss of plasma membrane integrity and the release of DAMPs. Many DAMPs that are released from dying cells in injured tissues elicit responses by signaling through TLR4 (19). Furthermore, TLR4 has been shown to play a role in promoting myocardial IRI (20). Therefore, we next set out to evaluate the role of TLR4 expression in cardiac grafts on neutrophil trafficking in reperfused cardiac grafts. Similar to our observations after treatment of heart recipients with Fer-1, in TLR4-deficient compared to wildtype hearts very few neutrophils adhered to the vessel walls, their density and extravasation was significantly reduced and their rolling velocity was significantly increased (Fig. 3A, B, E-G; Supplemental Videos 5, 6). TLR4 can signal via MyD88 or Trif. We have previously shown that neutrophils adhere to the vessel wall but fail to extravasate when heart grafts lack MyD88 expression (10). Thus, MyD88 signaling regulates a step in neutrophil
recruitment that is downstream from what we observed in TLR4-deficient grafts. Consequently, we investigated the role of Trif signaling in regulating the dynamic behavior of neutrophils in reperfused hearts. Transplantation of hearts that are deficient in CD14, a cell surface protein that mediates TLR4 internalization and activation of the Trif pathway or hearts that are deficient in Trif yielded results comparable to those in TLR4-deficient hearts (21) (Fig. 3 C-G; Supplemental Videos 7,8). To dissect whether TLR4 signaling on hematopoietic or non-hematopoietic graft cells regulates neutrophil recruitment to transplanted hearts, we generated bone marrow chimeras. Deletion of TLR4 in hematopoietic graft cells did not have any impact on neutrophil behavior. However, neutrophil adherence to the endothelium and extravasation into the myocardium was significantly impaired when TLR4 was deleted in non-hematopoietic cells (Supplemental Fig. 6; Supplemental Videos 9-11). Given that vascular endothelial cells and cardiomyocytes can signal through TLR4 we next used hearts from TLR4-floxed (TLR4\textsuperscript{fl/fl}) donor mice that were crossed to animals where expression of Cre is driven by an endothelial-specific receptor tyrosine kinase (Tie2) (Tie2-Cre/TLR4\textsuperscript{fl/fl}) or a cardiac-specific alpha myosin-heavy chain (Myh6) (Myh6-Cre/TLR4\textsuperscript{fl/fl}) promoter. Elimination of TLR4 on graft vascular endothelial cells, but not on cardiomyocytes, recapitulated the phenotype that we have observed in grafts that are globally deficient for TLR4 (Fig. 3 H-L; Supplemental Fig. 7; Supplemental Videos 12-14).

**Type I interferon signaling regulates early steps of neutrophil recruitment to transplanted hearts**
Trif-mediated signaling stimulates the production of Type I interferons, a process that is regulated by the transcription factors IRF3, IRF5 and IRF7 (22). Donor hearts that lacked expression of type I interferon receptor (IFNAR) or the combination of IRF3, IRF5 and IRF7 displayed increased neutrophil rolling velocity, failure of neutrophils to adhere to the vessel wall, and marked reductions in neutrophil extravasation into the myocardium compared to wildtype grafts (Fig. 4 A-C; F-H; Supplemental Videos 15,16). The neutrophil behavior in these grafts was similar to Fer-1-treated recipients as well as TLR4-, CD14- and Trif-deficient hearts. Consistent with the concept that ferroptosis regulates neutrophil recruitment through a type I IFN dependent pathway, administration of recombinant IFN-α restored neutrophil recruitment after transplantation into Fer-1-treated hosts (Fig. 4 D-H; Supplemental Video 17).

Neutrophilic infiltration of injured hearts mediates tissue damage

Having shown that ferroptotic cell death regulates the recruitment of neutrophils into injured myocardium we next set out to examine whether neutrophilic accumulation contributes to cardiac damage. We subjected B6 mice that lacked expression of TLR4 on endothelial cells (Tie2-Cre/TLR4fl/fl), TLR4fl/fl control mice and neutrophil-depleted B6 mice to myocardial IRI in an in vivo closed-chest model. We observed significant reductions in neutrophilic infiltration into injured Tie2-Cre/TLR4fl/fl, comparable to the levels seen in neutrophil-depleted animals (Fig. 5A, B). We found that either deletion of TLR4 in endothelial cells or depletion of neutrophils resulted in reductions in infarct size at 4 days after injury compared to controls (Fig. 5 C, D). Echocardiography showed improvements in LV ejection fraction, reductions in LV systolic volume and smaller
akinetic areas in Tie2-Cre/TLR4\textsuperscript{fl/fl} hearts as well as neutrophil-depleted mice compared to controls (Fig. 5 E-H).

**Discussion**

Mechanisms guiding leukocyte recruitment differ between various tissues and organs. Intravital two-photon imaging has enabled us to gain novel insights into cellular and molecular cues that regulate neutrophil recruitment into injured hearts (9). Our observations suggest that endogenous substances released during ferroptotic cell death trigger TLR4 signaling in endothelial cells. More generally, it is likely that ferroptosis results in the release of multiple DAMPs and alarmins that are recognized by a variety of cells within the heart, including immune cells, endothelial cells, and fibroblasts. Within endothelial cells, signaling through Trif promotes neutrophil adhesion to coronary veins in type I interferon-dependent fashion. The failure of neutrophils to adhere to the vessel wall in hearts that do not express TLR4, CD14, Trif, IFNAR or IRF 3/5/7 resembles our previous observations after inhibition of LFA-1 or CXCL2 (9, 10). Engagement of CXCR2 on the surface of neutrophils by CXCL2 presented on the surface of vascular endothelial cells changes the confirmation of the integrin LFA-1 thereby promoting their firm arrest. Interestingly, type I interferon signaling has been shown to contribute to the production of CXCL2 in models of kidney IRI (23). In contrast to the inability of neutrophils to adhere to the vessel wall when we inhibit ferroptosis or disrupt TLR4/Trif signaling in endothelial cells of injured hearts, we have previously reported that neutrophil recruitment is inhibited further downstream when TLR9/MyD88 signaling is eliminated in tissue-resident cardiac CCR2\textsuperscript{+} macrophages. Collectively, these observations indicate that leukocyte trafficking
in injured hearts is regulated through various cell populations that signal via different pathways in response to the release of various endogenous ligands.

Our findings extend previous observations regarding the role of TLR4 in promoting IRI in hearts and other organs. Kaczorowski demonstrated that the expression of inflammatory markers was decreased in transplanted hearts after neutralization of HMGB1, an endogenous TLR4 ligand that is released from dying cells, or after elimination of TLR4, CD14, MyD88 or Trif in both donor and recipient (24) (25). Similarly, in a model of myocardial IRI induced by transient coronary artery ligation, cardiac MPO expression, a surrogate marker for neutrophilic infiltration, is reduced when mice lack expression of TLR4 (26). Using an analogous experimental model, Li showed that heat shock protein 60, a DAMP that is released from ischemic myocardium, activates an inflammatory cascade through a TLR4/MyD88-dependent pathway (20). While these studies did not address the question which cells were activated through TLR4, a few reports have suggested a role for macrophages. Zhu proposed a cascade whereby DAMPs mediate the production of IL-23 by macrophages through TLR4-dependent signaling in transplanted hearts (27). IL-23 promoted the generation of IL-17+ γδ T cells, which resulted in the recruitment of neutrophils to the graft. TLR4 signaling in hematopoietic cells has also been suggested to mediate hepatic IRI (28). In a model of warm liver IRI, Zhai demonstrated that inflammation was mediated through TLR4 signaling in macrophages, which resulted in MyD88-independent IRF3-dependent production of type I interferons (29, 30).

The Kubes laboratory has published several reports examining how TLR4 and CD14 expression regulate neutrophil recruitment into the capillary beds of various organs
and tissues after local or systemic endotoxin challenges. Andonegui showed that TLR4 expression by endothelial cells regulates neutrophil sequestration into lung and brain capillaries following systemic administration of LPS (31) (32). Tissue-specific variations exist with regard to dependence on CD14 expression for leukocyte sequestration in response to LPS (33). To this end, unlike our observations in reperfused cardiac grafts, endotoxin-mediated neutrophil sequestration into the microcirculation of the central nervous system or liver was not dependent on CD14 expression (32, 34).

It has become widely accepted that non-apoptotic forms of cardiomyocyte cell death result in the release of alarmins and DAMPs that ultimately result in the initiation of inflammation, referred to as necroinflammation (7, 35). Various cell death signaling pathways have been considered to be involved in myocardial injury. A recent study demonstrated that inhibition of glutaminolysis, a pathway that can induce ferroptotic cell death, resulted in functional improvements in hearts subjected to IRI in an ex vivo preparation (36). Consistent with these observations, we have shown that treatment with Fer-1 reduced the abundance of several oxidized lipids that have been associated with ferroptosis and decreased the release of CK. However, the initial driver of cardiomyocyte death following IRI in vivo has not been conclusively identified. We have demonstrated that ferroptosis mediates early cell death and promotes the initial stages of inflammation following heart transplantation and also during myocardial IRI after transient occlusion of a coronary artery. Intriguingly, we found that cells within the myocardium are differentially responsive to ferroptosis. Cardiomyocytes and fibroblasts, but not endothelial cells, appeared to be most vulnerable to ferroptotic cell death. Ferroptosis has recently been described as a form of programmed cell death that has been implicated in various
pathologies including malignancies, neurodegeneration, and IRI involving the kidney and brain (37-41). Biochemically, ferroptosis is dependent on the generation of lipid peroxides, particularly hydroperoxides of polyunsaturated (arachidonoyl) phosphatidylethanolamines (42). Manipulations that interfere with the production of lipid peroxides result in resistance to ferroptosis, and, conversely, inhibition of cellular mechanisms that eliminate lipid peroxides sensitizes cells to ferroptosis (15, 43, 44). Many of the biochemical mechanisms essential for ferroptosis have been observed in the context of myocardial IRI (45). In addition, enzymatic activities required to execute ferroptosis are also important for cardiomyocyte cell death (46). Notably, a recent study has shown that inhibition of ferroptosis reduces cardiac dysfunction in a model of pressure overload-induced heart failure suggesting a role for ferroptotic cell death beyond the stages immediately following the injury (47).

Similar to our observations, treatment with Nec-1 has been shown to have a protective effect in models of heart IRI (48). However, as Nec-1 can inhibit both necroptosis and ferroptosis, no definitive conclusions about the cell death pathways could be drawn from these investigations. Previous studies using RIPK3-deficient mice have suggested that necroptotic cell death may play a role at later time points after heart injury (12). For example, RIPK3-deficient heart allografts demonstrated less cell death, attenuation in lymphocytic infiltrates and reduced levels of DAMPs 12 days after transplantation into allogeneic hosts (49). We suggest that necroptotic death of graft cells is triggered by tissue-resident or infiltrating immune cells releasing inflammatory mediators, such as TNF-α (49). This notion is supported by our demonstration that neutrophilic infiltration into injured hearts, a process that is triggered by ferroptosis,
augments cell death and mediates cardiac dysfunction. Similarly, following acute kidney injury, necroptosis has been shown to drive a second wave of cell death that amplifies the initial inflammation that is mediated by ferroptosis (50).

In addition to mediating primary graft dysfunction, neutrophils have been shown to enhance alloimmune responses. To this end, it has previously been shown that neutrophilic infiltration into cardiac allografts promotes the subsequent recruitment of alloreactive T cells (51). Notably, early graft infiltration by memory CD8+ T cells plays an important role in mediating rejection of cardiac allografts that are subjected to prolonged ischemic storage, a process that is known to be associated with enhanced neutrophilic graft infiltration (52). Furthermore, peri-operative depletion of neutrophils or inhibition of neutrophil chemotactic pathways synergizes with co-stimulatory blockade in extending the survival of cardiac allografts (51). Therefore, we speculate that inhibiting ferroptosis in the peri-operative period may attenuate alloimmune responses.

In conclusion, our findings provide support for the notion that ferroptotic cell death triggers initial inflammatory responses after heart transplantation. We suggest that inhibition of ferroptosis or targeting the TLR4/Trif/Type I interferon pathway may be viable clinical strategies to improve outcomes in heart transplant recipients and patients, who require reperfusion of ischemic myocardium after coronary occlusion. The recent development of platforms to perfuse and recondition organs ex vivo provides an avenue for the administration of pharmacological agents to donor grafts (53). Our findings suggest that treating donor hearts with a ferroptosis inhibitor prior to transplantation may reduce ischemic injury.
Methods

Mice. C57BL/6 (B6), B6 TLR4-deficient, B6 CD14-deficient, B6 Trif-deficient, B6 IFNAR (type I interferon receptor)-deficient, B6 Tie2-Cre and B6 Myh6-Cre mice were purchased from The Jackson Laboratories. B6 RIPK3-deficient mice were obtained from Genentech. B6 TLR4 floxed mice were provided by T. Billiar (University of Pittsburgh, Pittsburgh, PA, USA), B6 IRF 3/5/7 triple-deficient mice were provided by M. Diamond (Washington University in St. Louis). B6 LysM-GFP mice were provided by M. Miller (Washington University in St. Louis), who had originally obtained them from K. Ley (La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA). B6 RIPK3-deficient, B6 TLR4 floxed and B6 LysM-GFP mice were bred at our facility. B6 TLR4 floxed mice were intercrossed with B6 Tie2-Cre or B6 Myh6-Cre mice to generate animals that lacked expression of TLR4 on vascular endothelial cells and cardiomyocytes, respectively. Some recipients were treated with vehicle, necrostatin-1 (Nec-1) (4 mg/kg intravenously 1 hour before and intraperitoneally 2 hours after reperfusion) (Sigma, St. Louis, MO), ferrostatin-1 (Fer-1) (10 mg/kg intraperitoneally 1 hour before reperfusion) (Santa Cruz Biotechnologies, Dallas, TX), Z-VAD-FMK (10 mg/kg intraperitoneally 1 hour before reperfusion) (Santa Cruz Biotechnology, Dallas, TX), recombinant mouse interferon-alpha A Protein (10^4 units intraperitoneally 1 hour before reperfusion) (R&D Systems, Minneapolis, MN) or antime mouse Ly6G antibodies (500 μg intraperitoneally day -1, 250 μg intraperitoneally days 0, 1) (clone 1A8, Bio X Cell, West Lebanon, NH), as indicated for specific experiments. Six- to ten week old male and female mice were used for the studies. Gender-matched mice were used for the transplant procedures.
**Heart transplantation.** Cardiac grafts were harvested from wildtype B6 or various gene-deficient donor mice and transplanted into the right neck of B6 LysM-GFP or B6 CD45.1+ hosts as previously described (9).

**Bone marrow transplantation.** Bone marrow chimeras were generated as previously described (54). Briefly, bone marrow was harvested from the femora of donor mice and T lymphocytes were depleted via negative selection with anti-CD90-labeled magnetic microbeads. Recipient mice were lethally irradiated (10 Gy) and subsequently received 1 × 10⁷ T cell-depleted donor bone marrow via intravenous injection. Bone marrow chimeras were used as heart donors at least 90 days after the bone marrow transplant. We have previously reported that this regimen results in near complete replacement of hematopoietic cells in hearts (54).

**Ischemia Reperfusion Injury.** For closed-chest IRI, two to four-month old B6 mice were anesthetized with sodium pentobarbital, intubated, and mechanically ventilated. The heart was exposed through a midline incision and an 8-0 prolene suture was placed around the proximal left coronary artery to maximize the ischemic area. The suture was then threaded through a 1 mm piece of polyethylene tubing forming a loose snare to serve as the arterial occluder. Each end of the suture was exteriorized through the thorax and stored in a subcutaneous pocket. The skin was then closed over the exteriorized suture ends with 5-0 prolene suture. Instrumented mice were allowed to recover for 2 weeks prior to induction of ischemia. Ischemia was induced after anesthetizing the animals with inhaled 1.5% isoflurane. The skin was opened over the subcutaneous pocket and the exteriorized suture exposed. The suture ends were dissected away from the subcutaneous tissue and tension was exerted until ST-segment elevation was seen on
the ECG. Ischemia was confirmed by visualizing wall motion abnormalities using simultaneous echocardiography. Following 90 minutes of ischemia, tension was released and the suture ends were placed back into the subcutaneous pocket. The skin was then closed. Sham animals underwent the identical procedure with the exception that tension was not placed on the suture ends. Vehicle or Fer-1 (0.25 mg IP) was administered 24 and 2 hours before IRI.

**Echocardiography.** Mouse echocardiography was performed in the Washington University Mouse Cardiovascular Phenotyping Core facility using the VisualSonics 770 Echocardiography System. 2D and M-mode images were obtained in the long and short axis views. Ejection fraction and LV dimensions were calculated using edge detection software and standard techniques. The akinetic region was calculated by measuring the area of the akinetic portion of the LV myocardium and normalizing it to the area of the total LV myocardium. Measurements were performed on 3 independently acquired images per animal by investigators, who were blinded to experimental groups.

**Triphenyltetrazolium chloride (TTC) staining.** Hearts were harvested, perfused with 20ml of ice cold PBS, and sliced into 4 pieces using a razor blade. Myocardial slices were then immersed in 1% TTC (Sigma, St. Louis, MO) diluted in 0.9% sodium chloride and incubated from 30 minutes on an agitator at 37°C. Slices were then fixed in 10% formalin and imaged on a Zeiss stereomicroscope. Quantification of the infract area was performed using ImageJ software in blinded fashion.

**Immunostaining and Picrosirius Red staining.** For histological analyses, tissues were fixed in 2% PFA overnight at 4°C, dehydrated in 70% ethyl alcohol, and embedded in paraffin. 4-μm sections were cut and stained with Picrosirius red using standard
techniques. Picrosirius red staining was quantified using Image J software. For all immunostaining assays tissues were fixed in 2% PFA overnight at 4°C, embedded in OCT, infiltrated with 30% sucrose, frozen, and 12-μm cryosections cut. Primary antibodies used were: Ly6G (clone 1A8) 1:100 (BD Biosciences, San Jose, CA), cleaved Caspase-3 (clone 5A1E) 1:1000 (Cell Signaling). Immunofluorescence was visualized using appropriate secondary antibodies on a Zeiss confocal microscopy system. For all experiments, at least 4 sections from 4 independent samples were analyzed in blinded fashion.

Flow cytometry. Heart tissue was prepared for flow cytometry as previously described (10). Cells were stained with fluorochrome-labeled anti-CD45 (clone 30-F11, eBioscience), anti-CD31 (clone MEC13.3, BD Biosciences), Anti-Feeder Cells (clone mEF-SK4, Miltenyi Biotec, Bergisch Gladbach, Germany) (55), DAPI (4′,6-diamidino-2-phenylindole) (ThermoFisher Scientific) and isotype controls.

Neutrophil chemotaxis. Neutrophils were isolated from the bone marrow of B6 mice by negative selection as previously described (10). 10⁶ neutrophils, suspended in 100 μl complete RPMI medium containing 10% FBS, were placed in the upper chamber of a transwell (6.5 mm, 3-μm pore size, polycarbonate membrane, ThermoFisher Scientific). Recombinant CXCL2 (c452-M2-010; 100 ng/ml; R&D Systems, Minneapolis, MN) and/or Fer-1 (100 μM) were added to complete RPMI medium in the lower chamber. Neutrophil chemotaxis was assessed after a 2-hour incubation period at 37°C in 5% CO₂ by counting cells using a hemocytometer in 10 μl of complete RPMI medium that were collected from the bottom chamber.
Intravital two-photon microscopy. Intravital imaging of heart grafts was performed using a custom-built 2-photon microscope running ImageWarp version 2.1 acquisition software (A&B Software, New London, CT) as previously described (9). For time-lapse imaging of neutrophil trafficking in cardiac grafts, we averaged 15 video-rate frames (0.5 seconds per slice) during the acquisition to match the ventilator rate and minimize movement artifacts. Each plane represents an image of 220 × 240 μm in the x and y dimensions. Twenty-one sequential planes were acquired in the z dimension (2.5 μm each) to form a Z stack. Each individual neutrophil was tracked from its first appearance in the imaging window and followed up to the time point at which it dislocated more than 20 μm from its starting position. To visualize coronary vessels and determine whether neutrophils were intravascular or extravascular we injected 50 μl of PBS containing 15 μl of 655-nm nontargeted Q-dots (ThermoFisher Scientific) intravenously immediately prior to imaging.

To determine the percentage of extravasated neutrophils, the number of extravascular neutrophils was divided by the sum of intravascular and extravascular neutrophils. Multidimensional rendering and manual cell tracking was done with Imaris (Bitplane, Zurich, Switzerland). Data were transferred and plotted in GraphPad Prism 6.0 for creation of the graphs.

Mass spectrometric (MS) analyses of phosphatidylethanolamine and its oxidation products. Lipids were extracted by using the Folch procedure (56) and phosphatidylethanolamines (phospholipids) were analyzed by LC-MS/MS (15) using normal phase (Luna 3 μm Silica (2) 100Å, 150 x 2.0 mm, (Phenomenex) at a flow rate of 0.2 mL/min on a Dionex Ultimate 3000 HPLC system (ThermoFisher Scientific) at 35 °C. Gradient solvents A: propanol:hexane:water (285:215:5, v/v/v) and B:
propanol:hexane:water (285:215:40, v/v/v) containing 10 mM ammonium acetate were used. The column was eluted for 0-23 min with a linear gradient from 10% to 32% B; 23-32 min from 32 to 65% B; 32-35 min from 65 to 100% B; 35-62 min held at 100% B; 62-64 min from 100% to 10% B followed by and equilibration from 64 to 80 min at 10% B. MS and MS² analysis was performed on a Q-Exactive hybrid-quadrupole-orbitrap mass spectrometer (ThermoFisher Scientific) in negative ion mode at a resolution of 140,000 for the full MS scan and 17,500 for the MS² scan in a data-dependent mode. Analysis of data was performed using software package Compound Discoverer™ (ThermoFisher Scientific) with an in-house generated analysis workflow and oxidized phospholipid database. Phospholipids were filtered by retention time and confirmed by fragmentation analysis.

*Mass spectrometric analyses of eicosanoids, docosanoids, and oxidized metabolites of linoleic acid.* Mass spectrometric analyses of signaling eicosanoids, docosanoids, and oxlams were performed using a charge-switch strategy by derivatization with N-(4-aminomethylphenyl) pyridinium (AMPP) and subsequent LC-MS/MS with MRM and accurate mass determination of diagnostic product ions as previously described (57).

*IRI ex vivo (Langendorff preparations).* Hearts from vehicle control and Fer-1 (0.25 mg intraperitoneally injected 24 and 2 hours before harvest) treated B6 mice were isolated and perfused at a constant pressure of 70 mm Hg with modified Krebs-Henseleit buffer. After a 20-minute stabilization period, hearts were subjected to no-flow ischemia (t=0 minutes) for 30 minutes followed by reperfusion (t=30 minutes) for up to 60 minutes (t=90 minutes). To assess the degree of cardiomyocyte injury after IRI *ex vivo*, we measured creatine kinase (CK) release in the effluent collected every 5 minutes during reperfusion.
using a commercially available CK assay kit (Cat. #326-10; Sekisui Diagnostics, Lexington, VA) according to the manufacturer’s recommendations. CK activity was normalized for dry heart weight, and data are expressed as units per gram of cardiac tissue.

**Statistical analysis.** Differences between groups were compared by using two-sided Mann-Whitney U test. Multiple means were compared by using a one-way analysis of variance followed by post-hoc Tukey’s multiple comparison test. P<0.05 was indicative of a statistically significant difference. Data were analyzed by using software (Prism, version 6.0-7.0; GraphPad, La Jolla, CA) and are presented as dot plots or box whisker plots generated in PRISM. Statistical tests and exact sample sizes used to calculate statistical significance are stated in the appropriate figure legends.

**Study approval.** Animal experiments were approved by The Institutional Animal Studies Committee at Washington University. Animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by The National Academy of Sciences and published by The National Institutes of Health and with the Principles of Laboratory Animal Care formulated by The National Society for Medical Research.
Author contributions

W.L. performed the heart transplantation and intravital 2-photon imaging studies; J.M.G, A.H. and S.T. performed analysis of intravital 2-photon imaging studies; R.H. and D.R.P. performed flow cytometric analyses; H-M.H. performed chemotactic assays; G.F. and A.B. performed experiments in myocardial infarction model; S.E., I.L. and M.C. performed experiments involving Langendorff preparations; X.L. and R.G. performed mass spectrometric analyses of eicosanoids, docosanoids and oxidized metabolites of linoleic acid; Y.Y.T. and V.E.K. performed mass spectrometric analyses of phosphatidylethanolamine and its oxidation products; D.L.M., A.L. and A.E.G. assisted with experimental design and critical review of the manuscript; K.L. and D.K. are responsible for all aspects of this manuscript including experimental design, data analysis, and manuscript production.

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References


Figures and figure Legends

A

WT 02:00:00 02:15:00 02:30:00

B

Nac-1

C

RIPK3 KO

D

Fer-1

E

Rolling velocity (μm/s)

F

Cell density (mm²)

G

Extravasated cells (%)

H

PMNs arrival (cells/min)

I

Vehicle Fer-1

Ethidium bromide DAPI

J

Vehicle Fer-1

% DAPI

K

pmol PE (38.4)+0/μmol PE (38.4)

Vehicle Fer-1

ns

*
**Figure 1:** Ferroptosis regulates cell death and promotes neutrophil recruitment in injured hearts. Intravital two-photon imaging of neutrophil (green) behavior in (A) control wildtype cardiac grafts, (B) after treatment of recipient mice with Nec-1, (C) in RIPK3-deficient donor hearts and (D) after administration of Fer-1 to heart recipients. Vessels are labeled red after injection of quantum dots. n=4 per experimental group. (E) Intravascular rolling velocities of neutrophils, (F) density of neutrophils and (G) percentage of extravasated neutrophils in experimental conditions displayed in A-D. Data in (E)-(G) represent the mean ± s.e.m. ns = not significant, *p<0.05, **p<0.01, ***p<0.001 by one-way analysis of variance followed by post-hoc Tukey's multiple comparison test. (H) Neutrophil recruitment per minute to coronary veins in control heart grafts and after treatment of recipient mice with Fer-1. (I) Cardiomyocyte death determined by ethidium bromide in cardiac grafts after treatment of recipient mice with (left) or (right) Fer-1. n=4 per experimental group, 200X magnification. (J) Flow cytometric assessment of death (DAPI+) of (top) fibroblasts and (bottom) endothelial cells in B6 cardiac grafts after transplantation into vehicle- or Fer-1-treated syngeneic recipients. n=6 per experimental group. (K) LC/MS assessment of pro-ferroptotic PE molecular species. MS spectrum of PE (left) obtained from wildtype mice. Inset: MS spectrum in the range of m/z from 798.49 to 798.58. The content of PE(38:4)+OO molecular species (HOO-AA-PE, ferroptotic cell death signal) in cardiac grafts after treatment of recipient mice with vehicle or Fer-1. Data in (H)-(K) represent the mean ± s.e.m. ns = not significant, *p<0.05 by two-sided Mann-Whitney U test.
Figure 2: Ferroptosis regulates cardiomyocyte cell death and LV remodeling following myocardial infarction. (A) Measurement of total CK activity in control hearts and hearts subjected to 30 minutes of ischemia followed by 30 minutes of reperfusion using Langendorff preparations. Mice were treated with either vehicle control or Fer-1 2 hours prior to harvest. n=5 per group. (B) Serial measurement of CK activity following reperfusion in control hearts, vehicle- and Fer-1-treated hearts subjected to IRI. n=5 per group. *p<0.05 compared to control, **p<0.05 compared to other groups by two-sided Mann-Whitney U test. (C) Arachidonic acid metabolites measured by mass spectrometry (LC-MS/MS) in control hearts, vehicle- and Fer-1-treated hearts subjected to IRI. Data are displayed as box and whisker plots. Line indicates the mean value. *p<0.05 compared to control, **p<0.05 compared to other groups, #p<0.05 compared to vehicle IRI group. (D) TTC staining and measurement of infarct area in hearts obtained from vehicle- and Fer-1-treated mice 48 hours following 90 minutes of IRI. *p<0.05 compared to vehicle control. 10X magnification. (E) Ly6G immunostaining and quantification 48 hours following 90 minutes of IRI in vehicle- and Fer-1-treated hearts. Blue: DAPI. 200X magnification. *p<0.05 compared to vehicle control. (F) Echocardiography of vehicle- and Fer-1-treated mice 4 weeks following 90 minutes of IRI. Yellow dashed line denotes the akinetic area. (G-I) Quantification of LV ejection fraction, LV diastolic and systolic volumes, and akinetic area in vehicle- and Fer-1-treated mice 4 weeks following IRI. *p<0.05 compared to vehicle control. Akinetic area is expressed as a percentage of LV area. (J) Picrosirius red staining (red) and infarct size quantification 4 weeks following 90 minutes of IRI in vehicle- and Fer-1-treated hearts. Line represents the mean. ns = not
significant, *p<0.05 by two-sided Mann-Whitney U test. LV: left ventricle; Fer-1: ferrostatin-1.
**Figure 3:** Vascular endothelial TLR4 expression regulates neutrophil recruitment to injured hearts via Trif signaling. Intravital two-photon imaging of neutrophil (green) behavior in (A) control wildtype, (B) TLR4-deficient, (C) CD14-deficient and (D) Trif-deficient cardiac grafts. Vessels are labeled red after injection of quantum dots. n=4 per experimental group. (E) Intravascular rolling velocities of neutrophils, (F) density of neutrophils and (G) percentage of extravasated neutrophils in experimental conditions displayed in A-D. Intravital two-photon imaging of neutrophil (green) behavior in hearts that lack TLR4 expression on (H) vascular endothelial cells (Tie2cre-TLR4fl/fl) or (I) cardiomyocytes (Myh6cre-TLR4fl/fl). (J) Intravascular rolling velocities, (K) density of neutrophils and (L) percentage of extravasated neutrophils in experimental conditions displayed in E, F as well as control TLR4 floxed heart grafts. Data in (E)-(G) and (J)-(L) represent the mean ± s.e.m. ns = not significant, *p<0.05, **p<0.01, ***p<0.001 by one-way analysis of variance followed by post-hoc Tukey's multiple comparison test.
Figure 4: Ferroptosis promotes neutrophil recruitment to injured hearts through type I interferon signaling. Intravital two-photon imaging of neutrophil (green) behavior in (A) control wildtype, (B) type-I interferon receptor (IFNAR)-deficient and (C) IRF3/5/7-triple-deficient heart grafts as well as after transplantation into Fer-1-treated recipients that received (D) vehicle or (E) recombinant type-I interferon at the time of implantation. Vessels are labeled red after injection of quantum dots. n=4 per experimental group. (F) Intravascular rolling velocities, (G) density of neutrophils and (H) percentage of
extravasated neutrophils in experimental conditions displayed in A-E. Data in (F)-(H) represent the mean ± s.e.m. ns = not significant, *p<0.05, **p<0.01, ***p<0.001 by one-way analysis of variance followed by post-hoc Tukey’s multiple comparison test.
Figure 5: Neutrophilic infiltration contributes to cardiac damage. (A) Ly6G immunostaining and (B) quantification 4 days following 90 minutes of close chest IRI in
hearts of control (TLR4\textsuperscript{fl/fl}), Tie2-Cre/TLR4\textsuperscript{fl/fl} and neutrophil-depleted (Ly6G Ab) mice. Blue: DAPI, red: Ly6G, scale bar: 40 μm. (C) TTC staining and (D) measurement of infarct area in hearts obtained from control (TLR4\textsuperscript{fl/fl}), Tie2-Cre/TLR4\textsuperscript{fl/fl} and neutrophil-depleted (Ly6G Ab) mice 4 days following 90 minutes of close chest IRI. 10X magnification. (E) Echocardiography of control (TLR4\textsuperscript{fl/fl}), Tie2-Cre/TLR4\textsuperscript{fl/fl} and neutrophil-depleted (Ly6G Ab) mice 4 days following 90 minutes of IRI. Yellow dashed line denotes the akinetic area. (F-H) Quantification of LV ejection fraction, LV diastolic and systolic volumes, and akinetic area in control (TLR4\textsuperscript{fl/fl}), Tie2-Cre/TLR4\textsuperscript{fl/fl} and neutrophil-depleted (Ly6G Ab) mice 4 days following 90 minutes of IRI. Akinetic area is expressed as a percentage of LV area. n=4-5 per experimental group. Lines represent the mean. ns = not significant, *p<0.05, **p<0.01 by one-way analysis of variance followed by post-hoc Tukey’s multiple comparison test. LV: left ventricle.