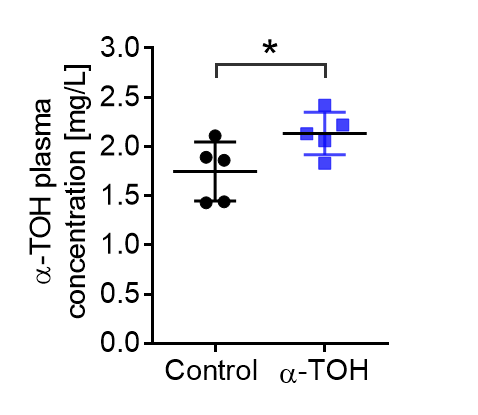
**Supplemental Material to the manuscript entitled:**

**α-Tocopherol Preserves Cardiac Function by Reducing**

**Oxidative Stress and Inflammation in Ischemia/Reperfusion Injury**

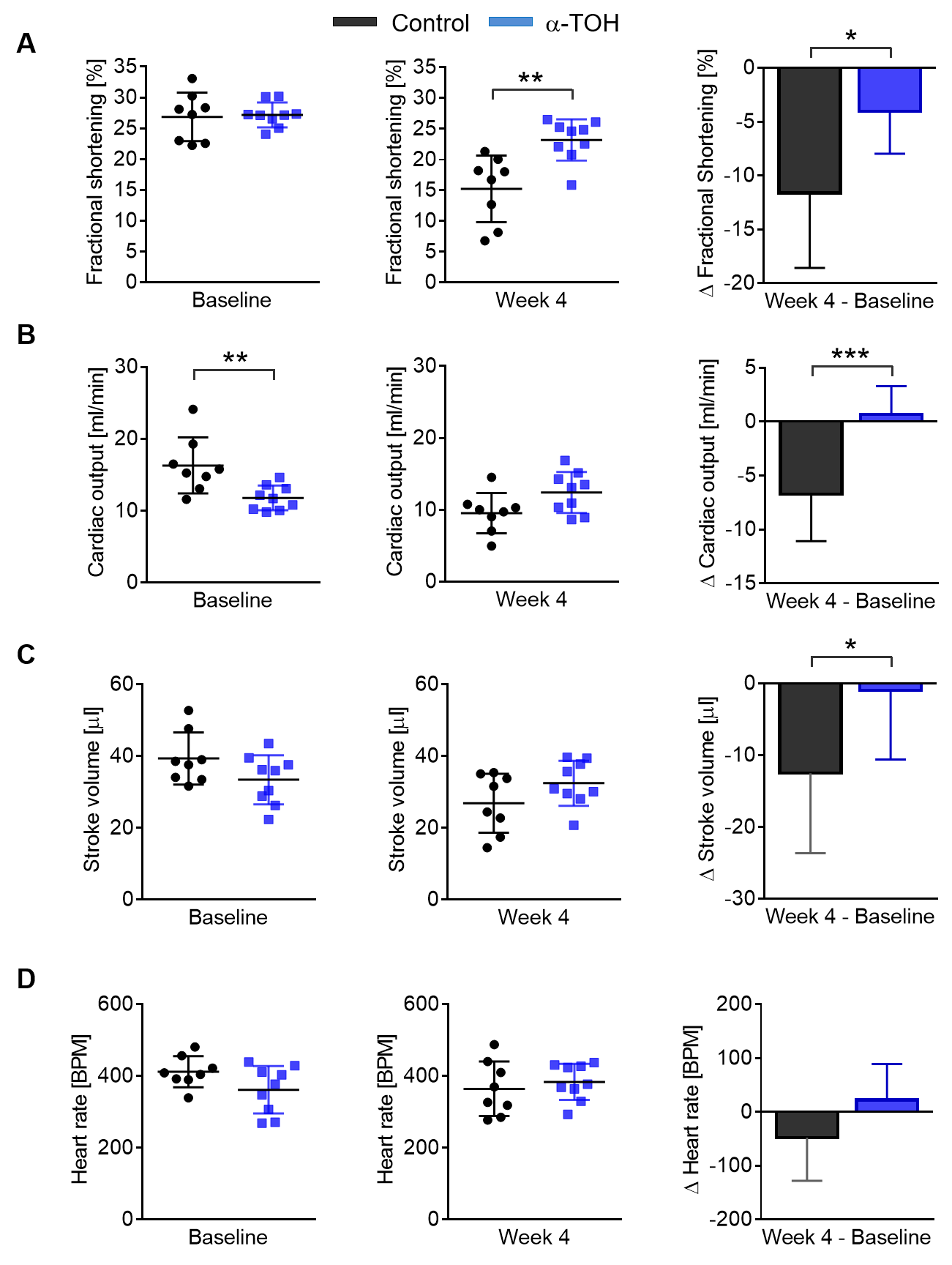
**Supplemental Figures**

**Supplemental Figure S1**

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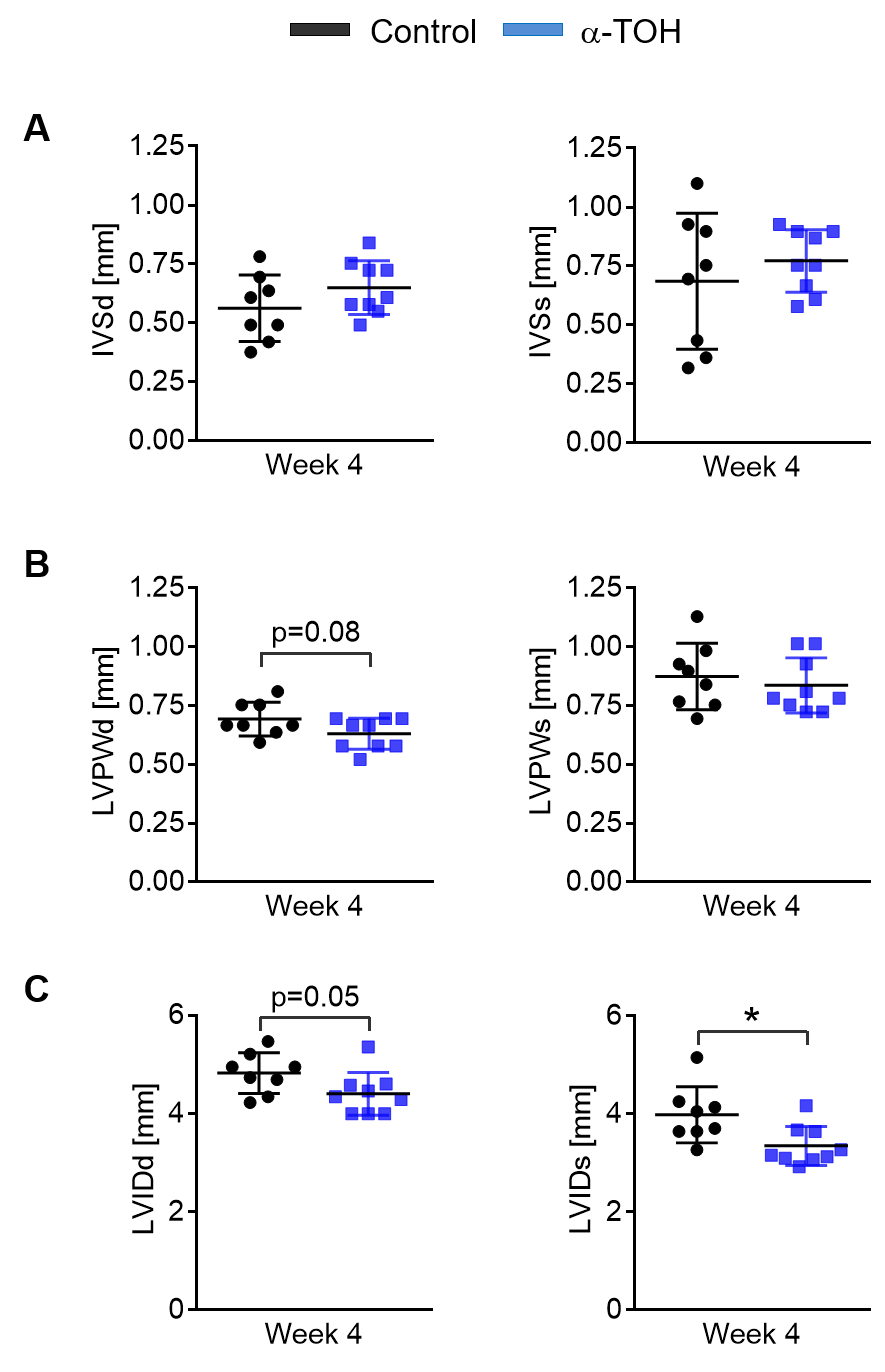
**Supplementary Figure S1: α-TOH plasma concentration in C57BL/6 mice.** α-TOH concentration of plasma collected from mice supplemented with 2.5 mg α-TOH/kg BW twice per day for three consecutive days compared to vehicle is significantly increased; n=3-4, \*\*p<0.01. Measurement of α-TOH was performed using liquid chromatography NEXERA X2-LC-30AD (Shimadzu, Duisburg, Germany).

**Supplemental Figure S2**

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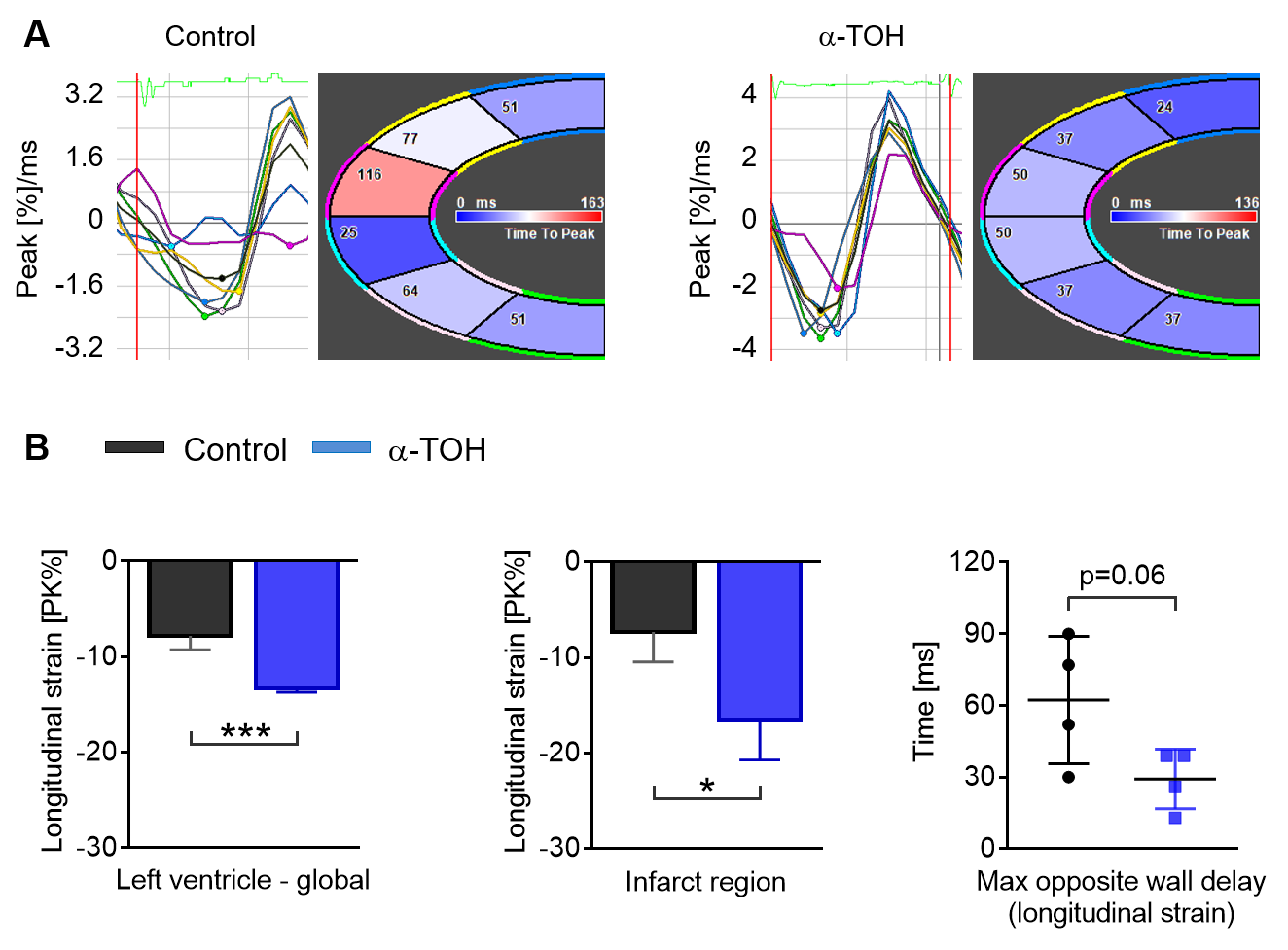
**Supplemental Figure S2: α-TOH preserves cardiac function.** Comparison of data assessed at baseline and 4 weeks post-I/R injury. **A)** Fractional shortening is increased in α-TOH-treated mice compared to controls; n=8–9, \*p<0.05 and \*\*p<0.01. **B)** Cardiac output is significantly decreased in control mice, as compared to α-TOH-treated mice (n=8–9, \*\*\*p<0.001); whereas **C)** stroke volume shows a significant decrease; n=8–9, \*p<0.05. **D)** No difference in heart rate between the treated groups can be observed; n=8–9.

**Supplemental Figure S3**

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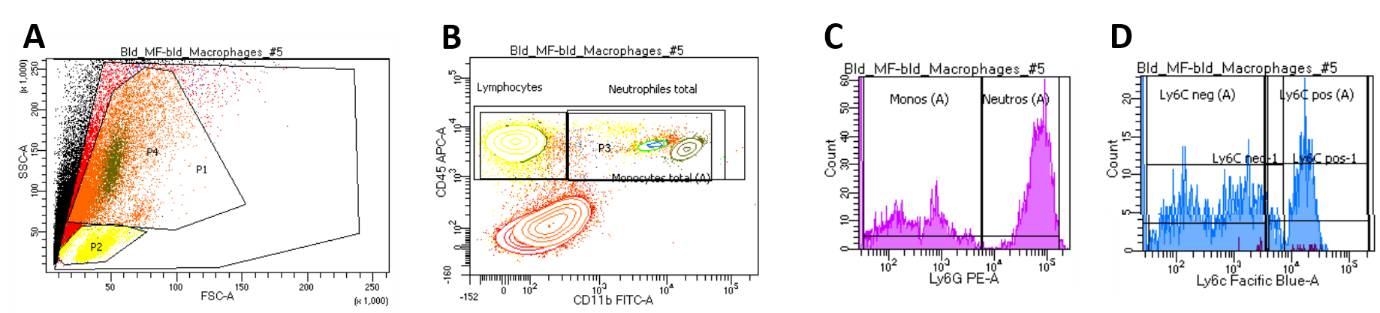
**Supplemental Figure S3: Left ventricle wall dimensions. A)** Echocardiographic measures of LV interventricular septal wall at end diastole (IVSd) and end systole (IVSs); no differences between treatment groups are observed. **B)** Echocardiographic measures of LV posterior wall end diastole (LVPWd) and LV posterior wall end systole (LVPWs); no differences between treatment groups are observed. **C)** Echocardio­graphic measures of LV internal diameter end diastole (LVIDd) and LV internal diameter end systole (LVIDs); decreases in LVIDd and LVIDs in α-TOH group as compared to controls at 4 weeks are observed; n=8–9, \*p<0.05.

**Supplemental Figure S4**



**Supplemental Figure S4: α-TOH-treatment improves longitudinal strain. A)** Representative images of longitudinal strain curves obtained from VevoStrain analysis software show strain measurements over time. Colored lines represent 6 standard myocardial regions; 7th black line calculates average (global) strain at each time point. Representative images of longitudinal strain time to peak show delays (light and red sections) in control animals, but not α-TOH-treated animals. **B)** Longitudinal strain analysis obtained similar differences to radial strain analysis; n=4.

**Supplemental Figure S5**

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**Supplementary Figure S5: Gating strategy for flow cytometry analysis.** Determination of total monocyte/macrophage, neutrophil and lymphocyte population **(A)** size and granularity-dependent and **(B)** are divided using CD45 and CD11b surface marker staining. **(C)** CD45+/CD11b+/Ly6G+ stained cells are further assigned as neutrophils and CD45+/CD11b+/Ly6G- as monocytes, whereas **(D)** sub-populations of Ly6Chigh and Ly6Clow monocytes are divided using Ly6C marker. Analysis is performed using BD FACS DIVA software version 8.0.1.

**Supplemental Methods**

**Study design**

Sample size of 10 mice per treatment group was defined using power analysis (power: 0.8, alpha: 0.05, expected difference: 30% ejection fraction (EF), mean: 39±9.5%) for ejection fraction as our major endpoint. Ultrasound analyses of EF were used as data inclusion/ exclusion criteria. Long-term experiments (4 weeks) were performed to study changes in cardiac function and infarct size using echocardiography and histology, respectively. In addition, short-term studies (1 and 3 days) were conducted to elucidate the mechanism of action of α-TOH by analyzing systemic cell distribution as well as mRNA expression profiles, myeloperoxidase activity, neutrophil infiltration, level of ROS, lipid distribution, and lipid peroxidation in the infarcted myocardium.

**Myocardial ischemia/reperfusion injury in mice**

After anesthesia, mice were orally intubated and ventilated using a rodent ventilator (Model 687, Harvard Apparatus), with a tidal volume of 0.18 ml at 120 breaths/min. Mice underwent open-chest surgery to induce left coronary artery occlusion (CAO) for 60 min, followed by reperfusion. All operations were carried out in a blinded manner such that the surgeon was blinded to the treatment administered. Randomized mice were IP injected with either a vehicle or α-TOH (2.5 mg/kg BW in 0.8% DMSO; Sigma-Aldrich) 2 h prior to surgery, immediately after reperfusion, and twice per day for three consecutive days.

Following surgery, mice were culled at three different time points for respective analysis. The first group (n=9) was culled after one day for the analysis of ROS formation, MPO activity, and blood cell counts. The second group (n=10) was culled three days post-surgery for blood cell count measurements, and the determination of regulated oxidative stress genes, chemokines, cytokines, oxidized lipids, and infiltrated cells within the infarcted myocardium. The last group (n=10) underwent echocardiography prior to surgery and weekly post–I/R injury using a Vevo2100 high-frequency small-animal scanner (VisualSonics) equipped with a 22-55 MHz MS550D transducer, as well as Evans blue/TTC staining 28 days after intervention as described previously [34] and in the online-only Data Supplement. We registered 5 deaths out of 78 mice throughout the study: 3 control mice and 2 mice treated with α-TOH. Therefore, no correlation of mortality with the treatment was observed.

**Echocardiography**

Mice underwent serial echocardiography at baseline and weekly post-ischemia/reperfusion (I/R) injury using a Vevo2100 high-frequency small-animal scanner (VisualSonics) equipped with a 22-55 MHz MS550D transducer. In brief, animals were placed under light sedation (range of 1% to 2% isoflurane) on the heated VisualSonics imaging station. The temperature of the animal, its electrocardiogram, and its breathing was monitored. During each echocardiographic examination, the parasternal long-axis and parasternal short-axis views of the heart were obtained. Echocardiography as well as video and image analyses were performed by a blinded investigator using the VisualSonics imaging software Vevo LAB 3/1/0. The ejection fraction (EF) was calculated using the Simpson method from the parasternal long-axis B-mode images. Fractional shortening (FS) as well as the dimensions of the left ventricular (LV) interventricular septal (IVS), LV internal diameter (LVID), and LV posterior wall (LVPW) at end diastole (d) and end systole (s) were calculated from the parasternal short-axis B-mode images. Radial and longitudinal strain analyses were conducted using a speckle-tracking algorithm provided by VevoStrain and VisualSonics imaging software Vevo LAB 3/1/0 by the same experienced investigator as previously described [34].

**Evans blue/TTC staining**

Mice were anesthetized 28 days post-I/R injury and the ischemic area (area at risk (AaR)) and infarcted area (infarct size (I)) were assessed by Evans blue/TTC staining. The left anterior descending (LAD) artery was re-ligated with the original suture, and 4% Evans blue (AppliChem) was injected to stain the perfused regions blue. The heart was then cut into six transversal slices which were stained with 1% TTC (Sigma-Aldrich) for 10 min at 37 °C in darkness. TTC turns the metabolically active areas red, while the infarcted necrotic myocardial tissue remains white. Then the heart slices were scanned on both sides using a high-resolution scanner (Epson Perfection Photo Scanner V370). A blinded researcher determined the infarct size by quantitative morphometric planimetry using the Image Pro Plus version 7.0 analysis software.

**Histology**

Mice were euthanized after three days, and the hearts were harvested and fresh-frozen in OCT Tissue Tec (Sakura® Finetek) compound using liquid nitrogen-cooled isopentane, followed by cutting into 6 µm sections (Microm HM 525 Cryostat, Thermo Fisher Scientific). Oil Red O (ORO, Sigma-Aldrich) was used to detect neutral lipid content in fresh-frozen samples. Sections were fixed in 10% formalin for 4 min, then washed in 60% isopropanol for 25 s. ORO was diluted in 100% isopropanol, further diluted with distilled H2O (3:2), and filtered. Using this working solution, samples were stained for 1 h followed by differentiation in 60% isopropanol. After a single washing step, Mayer’s hematoxylin dye was applied for 45 s and samples were washed again. To ensure the longevity of the lipid staining, samples were mounted in a water-soluble mounting medium, Aquatex (Merck Millipore).

**Immunofluorescence**

Cardiac sections taken at day 3 post-I/R injury were stained using rat anti-mouse Ly6G (Gr-1) monoclonal antibody (eBioscience) or a rat IgG isotype control (Thermo Fisher Scientific). An Alexa Fluor 546-labeled anti-rat antibody (Life Technologies) was applied for 30 min at RT followed by counterstaining with Hoechst 33342 nucleic acid dye (Thermo Fisher Scientific) for 5 min at RT. Sections were mounted with ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific) and further imaged using the Nikon A1r Plus Confocal Microscope and a 20x objective. For the staining and imaging procedures, samples were kept in darkness.

**Lipid extraction and analysis**

Prior to lipid extraction, samples were randomized and blinded. Lipids were isolated using a single-phase chloroform:methanol extraction as previously described [32]. In brief, the infarct area of the mice cardiac tissue samples (36-50 mg) were supplemented with the anti-oxidant butylhydroxytoluene (BHT, 1 µl of 100 mmol/l in ethanol) and PBS (pH=7.5) to avoid further oxidation. Cardiac tissue was mechanically disrupted and homogenized for 10 s using an electric homogenizer at an amplitude of 25 arbitrary units. Protein concentrations were determined using BCA protein assay (Pierce Biotechnology). Samples (10 µl) were extracted in a single-phase extraction with 20 volumes of CHCl3:MeOH (2:1) and 10 µl of an internal standard mix (in CHCl3:MeOH (1:1)) containing between 50 and 1000 pmol each of 23 non-physiological or stable isotope-labeled lipid standards.

Lipid analyses were performed by liquid chromatography electrospray ionization-tandem mass spectrometry using an Agilent 1290 HPLC coupled to an Agilent 6490 triple-quadrupole mass spectrometer with settings as follows: gas temperature 150 °C, gas flow 17 l/min, nozzle pressure 20 psi, sheath gas temperature 200 °C, sheath gas flow 10 l/min, capillary voltage 3500 V, nozzle voltage 1000 V. Liquid chromatography was performed on a Zorbax Eclipse Plus C18, 1.8 µm, 100 x 2.1 mm column (Agilent Technologies) using solvents A and B consisting of water:acetonitrile:isopropanol, 50:30:20 (v/v/v), and 1:9:90 (v/v/v) respectively, both containing 10 mM ammonium formate. The column was heated to 45 °C and the autosampler was regulated to 25 °C.

Lipid extracts (1 µl) were injected and separated under gradient conditions with a flow rate of 400 µl/min: 15% B to 50% B over 2.5 minutes, increased to 57% B over 0.1 minutes, increased to 70% B over 6.1 minutes, increased to 93% B over 0.1 minutes, increased to 96% B over 1.9 minutes, increased to 100% over 0.1 minutes, and held at 100% B for 0.8 minutes. The solvent was then decreased to 15% B over 0.1 minutes, held at 15% B for 0.9 minutes, increased in flow rate to 600 µl/min over 0.1 minutes, held at 600 µl/min 15% B over 1 minute, reduced in flow to 400 µl/min over 0.1 minutes, and held at 400 µl/min, 15% B until the next injection at 15.4 minutes. The first minute and final 2.3 minutes of each analytical run were diverted to waste.

The oxidized lipid species PC (16:0-13-HODE) and PC (16:0-9-HODE) were measured using a targeted lipidomics method combined with a dynamic multiple-reaction monitoring (dMRM) to collect data for a retention-time window specific to each lipid species. Results from the chromatographic data were analyzed using Mass Hunter Quantitative analysis version B.07, where relative lipid abundances were calculated by relating each area under the chromatogram for each lipid species to the corresponding internal standard (PC(13:0/13:0); Avanti Polar Lipids, Alabaster, US). Optimized dMRM transitions for oxidized PC were selected according to Aoyagi et al.[76], results were expressed as pmol/mg of heart tissue. Values for each lipid class were calculated as the sum of the individual lipid species.

**α-TOH measurement**

Murine plasma samples (100 µl) were protected from oxidation using 1 % ascorbic acid and 2.5 % butylated hydroxytoluene dissolved in ethanol (w/v). For extraction of lipid compounds a plasma/anti­oxidant solution was mixed with hexane and centrifuged for phase separation. Supernatant was collected and dried in a vacuum concentrator plus (1 h at 45 °C, Eppendorf, Hamburg, Germany), before samples were dissolved in 100 µl methanol/H2O (85:15, v/v). 10 µl of the suspended sample was injected in a liquid chromatography NEXERA X2-LC-30AD (Shimadzu, Duisburg, Germany). Tocopherols and tocotrienols were separated using a Phenomenex Kinetex 2.6 µM PFP column (2.6 µm, 150 x 4.6 mm), an isocratic flow of methanol/H2O (85:15 v/v) with a flow rate of 0.8 ml/min and detected using a fluorescent detector (excitation 296 nm; emission 325 nm).

**Synthesis and preparation of a ROS-sensitive nanoparticle**

An amphiphilic conjugate based on chlorin e6, luminol, and polyethylene glycol (PEG) was synthesized by conjugating both luminol and PEG with chlorin e6 using a standard 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) / N-hydroxysuccinimide (NHS) activated condensation reaction. Briefly, 100 mg (0.16 mmol) chlorin e6 was dissolved in 20 ml anhydrous dimethyl sulfoxide (DMSO), into which EDC (472 mg, 2.46 mmol) and NHS (288 mg, 2.5 mmol) were sequentially added. The reaction mixture was stirred at 50 °C in darkness for 20 h. Then luminol (56 mg, 0.32 mmol) was added to the activated solution. After 3 days, PEG-NH2 (640 mg, 0.32 mmol) was added. The reaction was conducted at 50 °C in darkness for 4 days. Finally, the reaction mixture was dialyzed (MWCO: 3500 Da) against deionized water to remove unreacted reagents and by-products. After filtration through a 0.22 μm syringe filter, the obtained aqueous solution was lyophilized to give rise to a dark-green powder that is defined as CLP (Classification, Labeling and Packaging under regulation 1272/2008). Prior to the experiment, the CLP nanoparticle was dissolved in PBS to a final concentration of 1 mg/ml. This ROS-sensitive nanoparticle (5 µg/g BW) was injected into I/R-injured mice 20 min prior to the mice being euthanized.

**References**

[76] R. Aoyagi, K. Ikeda, Y. Isobe, M. Arita, Comprehensive analyses of oxidized phospholipids using a measured MS/MS spectra library, J. Lipid Res. 58 (2017) 2229–2237. doi:10.1194/jlr.D077123.