# SUPPLEMENTAL APPENDIX

## Supplemental Methods

### Animal experimental protocol

Animal care and interventions were provided in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals, and all animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Heart failure with preserved ejection fraction (HFpEF) was induced in male C57BL/6J mice by feeding high fat diet (HFD, 60 kcal% fat, Research Diet, New Brunswick, NJ) starting at the age of 6 weeks, as we have done previously (1). Female mice were excluded because they are less susceptible to HFD-induced metabolic disturbances and inflammation (2). HFD mice were purchased from Jackson Laboratories (Bar Harbor, Maine) at the age of 24-26 weeks old. The HFD protocol was initiated at Jackson Laboratories at the age of 6 weeks and was continued after the purchased HFD mice arrived at our facility.

At the age of 25-27 weeks old, HFD mice were randomly assigned to three different treatments: 1) MitoTEMPO, (2-(2,2,6,6-tetramethyl-piperidin-1-oxyl-4-ylamino)-2-oxoethyl-triphenylphosphonium chloride, Enzo Life Sciences, Farmingdale, NY), daily intraperitoneal injection of 1 mg/kg for 2 weeks to scavenge mitoROS; 2) Interleukin (IL)-1 receptor antagonist (IL1RA, BioLegend, San Diego, CA), daily intraperitoneal injection of 3 mg/kg for 2 weeks to suppress IL-1β; 3) A bolus intravenous injection of 0.25 mL clodronate liposomes (FormuMax Scientific Inc, Sunnyvale, CA) followed by 0.3 mL clodronate liposomes intraperitoneal injection

bi-weekly for 2 weeks to deplete macrophages. Mice receiving USP sterile water or plain liposome injection were used as controls.

Fatty acid binding protein 4 (FABP4) knockout (KO) and wildtype (WT) mice, on C57BL/6J background, were kindly sponsored by Dr. David Bernlohr at the University of Minnesota. The male FABP4 KO and WT mice were started on HFD at 6 weeks of age and were studied at the age of 19-21 weeks. Age- and sex-matched WT mice fed with normal chow (Harlan, Indianapolis, IN) were used as controls.

**Fasting glucose and plasma insulin measurements**

Mice were fasted for six hours, and blood samples were collected from the tail vein. The fasting glucose level was measured by a glucometer (ACCU-CHEK, Roche Applied Science, Indianapolis, IN). Plasma was separated by centrifugation at 12000 rpm for 10 min at 4°C. The plasma fasting insulin was measured using Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Elk Grove Village, IL). The insulin resistance index, Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), was calculated as: fasting glucose (mM) × fasting insulin (mIU/L) / 22.5 (3).

**Non-invasive evaluation of cardiac function**

Echocardiography was performed using the Vevo 2100 (VisualSonics, Toronto, Canada) ultrasound system as in previous studies (4). Mice were anesthetized with 1-2% isoflurane in oxygen at 1 L/min. The electrocardiogram (ECG), respiration, and rectal temperature were continuously monitored with an integrated physiology platform. Body temperature and heart rate were maintained at 37-38 °C and above 400 bpm, respectively. B-mode images along the left ventricular parasternal long axis and then M-mode images at the mid-papillary level were obtained to calculate ejection fraction. Percent left ventricular ejection fraction (EF) was calculated as stated

previously. E/E' was assessed in the subcostal 4-chamber view by pulsed-wave and tissue Doppler imaging to evaluate diastolic function. Measurements were averaged from five consecutive beats during expiration.

**Invasive assessment of DD**

The Mikro-Tip (1.4 Fr) catheter (SPR-671, Millar, Houston, TX) was used to assess *in vivo* left ventricular pressure. In brief, mice were anesthetized with 1-2% isoflurane. The catheter as inserted into right common carotid artery and advanced into left ventricle. The pressure signal was recorded with Powerlab system (ADInstruments, Colorado Springs, CO) and analyzed with LabChart 7.3.8 Software (ADInstruments). The respiration, heart rate and rectal temperature were continuously monitored during the procedure.

**Inflammatory cytokines measurements**

Immortalized FABP4 KO and WT mouse macrophage cell lines (5) were grown to near confluence in RPMI 1640 medium with 10% fetal bovine serum and 10 ng/mL macrophage colony-stimulating factor. Supernatants were collected for IL-1β measurement after 1 hour treatment with 500 ng/mL LPS and 2 mM adenosine triphosphate (ATP). Mouse hearts were perfused with ice cold PBS before collection, and approximately 25 mg of heart tissue was homogenized in lysis buffer and the supernatant was collected. IL-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β levels were determined by commercially available ELISA kits (Mouse IL-1 beta/IL-1F2 Quantikine ELISA Kit, R&D Systems, Minneapolis, MN; Mouse IL-6 ELISA Kit, Mouse IL-10 ELISA Kit, Mouse TNF-α ELISA Kit, Mouse TGF-β ELISA Kit, Thermo Fisher Scientific, Waltham, MA).

**Protein Western blots**

Proteins were isolated from left ventricles and separated on a 4-20% SDS-PAGE gel and transferred onto 0.2 μm polyvinyl difluoride membranes. Following blocking in 5% BSA/PBS for 1 h, the membranes were incubated overnight with monocyte chemoattractant protein 1 (MCP-1) antibody (Cell Signaling Technology, #2029S, Danvers, MA) at 4°C followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA). Vinculin (Cell Signaling Technology) was used as a loading control. Optical density of the bands was analyzed with ImageJ Software.

**Isolation of cardiomyocytes and cardiac interstitial cells (non-cardiomyocyte cells)**

Cardiac interstitial cells were isolated as described before (4). Briefly, hearts were excised under isoflurane (2%) anesthesia and perfused with buffer (in mM: 113 NaCl, 4.7 KCl, 0.6 $Na_2HPO_4$, 0.6 $KH_2PO_4$, 1.2 $MgSO_4$, 0.032 Phenol Red, 12 $NaHCO_3$, 10 $KHCO_3$, 10 HEPES, 30 Taurine, 10 2-3-butanedione monoxime) for 7 min at 3 mL/min flow rate using a temperature controlled Langendorff perfusion system, followed by collagenase II perfusion (0.8 mg/mL, Worthington Biochemical Co. Lakewood, NJ) for 10 min at 37°C. Hearts were gently cut into small pieces and dissociated into single cells by pipetting. Cardiomyocytes were separated from interstitial cells by settling for 10 min. The cell pellet was collected for mitoROS measurement, and the suspension was prepared for flow cytometry to characterize macrophages.

**Flow Cytometry**

Cardiac macrophages were characterized by flow cytometry in a BD FACSAriaII (BD Biosciences, San Diego, CA) using the FACSDiva software (BD Biosciences). Cardiac interstitial cells isolated from mouse heart were incubated with antibodies (APC-Cy7 anti-CD11b, Invitrogen, #A15390, Carlsbad, CA; PE anti-F4/80, Invitrogen, #12-4801-82; FITC anti-CCR2, R&D Systems, #FAB5538F; APC anti-Timd4, BioLegend, #130022; Alexa Fluor® 647 anti-CD206,

BD Biosciences, #565250; APC anti-CD86, Invitrogen, #17-0862-82), all at 1 μL per 1 million cells at room temperature for 45 min. Propidium iodide (PI) (1 μg/mL, Invitrogen, P3566) was added to identify dead cells. Doublet cells and dead cells were gated out. Macrophages were gated on $CD11b^{+}F4/80^{+}$ (6,7), and the subpopulations were further gated on CD206, CD86, C-C motif chemokine receptor 2 (CCR2), and T cell immunoglobulin and mucin domain containing 4 (Timd4) respectively. Fluorescence minus one control was applied to guide gating. Data was analyzed with FlowJo Software (FlowJo, Ashland, OR) and the percentage of cells in alive single cell gate or $CD11b^{+}F4/80^{+}$ gate was reported.

**Microarray**

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) from the FABP4 KO and WT macrophages cell lines. cDNA synthesis was performed using iScript (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Microarray was performed using Mouse Inflammatory Response & Autoimmunity RT2 Profiler PCR Arrays (Qiagen, Hilden, Germany).

**Mitochondrial reactive oxygen species (mitoROS) measurement**

MitoROS was measured in the isolated ventricular cardiomyocytes by an inverted confocal laser scanning microscope (Olympus Life Science Solutions Americas Corp., Waltham, MA) as described previously (8). Briefly, isolated cardiomyocytes were resuspended in a minimal Eagle's medium (MEM) containing 5% fetal bovine serum, 1% penicillin/streptomycin, 1 mM pyruvate, 5.5 mM glucose, and 1% insulin-transferrin-selenium (ITS) with serially increasing $Ca^{2+}$ concentrations (0.2, 0.5, and 1 mM), and treated with 1 ng/mL IL-1β (R&D Systems, #401-ML-005, Minneapolis, MN) for 30 min at room temperature followed by MitoSOX Red incubation (5 μM; Thermo Fisher Scientific, Waltham, MA) for 10 min at 37°C in a 95%/5% $O_2/CO_2$ incubator. MitoSOX was excited by laser at 514 nm. The digital images were taken at 2048 × 2048 pixels.

Images were captured using 40× objective lens, and optical sections were <1 μm. The MitoSOX Red intensity was analyzed with ImageJ Fiji Software.

**Supplemental References**


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