Molecular Imaging Visualizes Recruitment of Inflammatory Monocytes and Macrophages to the Injured Heart

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Materials and Methods

Chemicals. The ECL1i peptide (LGTFLKC) was synthesized from D-form amino acids by CPC Scientific (Sunnyvale, CA). Maleimido-mono-amide-DOTA was purchased from Macrocyclics, Inc (Dallas, TX). DOTA-ECL1i was synthesized as reported.[1] ^{68}Gallium, ^{68}Ga ($t_{1/2}$ = 68 min; β^+=89%; E_{max} = 1.92 MeV) was obtained from the GalliaPharm® ^{68}Ge/^{68}Ga Generator and Modular-Lab system from Eckert & Ziegler Radiopharma GmbH. Ultra-pure 0.1 M HCl (15 mL) was used to elute $^{68}GaCl_3$. The eluate was passed through the Strata X-C cation exchange cartridge (Phenomenex®, 30 mg/mL). The trapped $^{68}GaCl_3$ was then released from the resin with 0.02 M HCl/98% acetone (0.8 mL). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and used as received. Water with a resistivity of 18.2 MΩ·cm was prepared by a Milli-Q® Integral 5 Water Purification System (Bedford, MA).

Characterization of ^{68}Ga-DOTA-ECL1i. Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on an Agilent 1200 Series with photodiode array detector and an ORTEC® scintillation photon counter. Both RP- and radio-HPLC analysis were performed using VYDAC® (Grace) 218TP54 (C_{18}, 5 μm, 4.6 mm i.d. × 250 mm) with a mobile phase of A: water (0.1 % TFA) and B: acetonitrile (0.1 % TFA), using a gradient of 0–90 % acetonitrile over 10 min with a 2 mL/min flow rate.

Radiolabeling of DOTA-ECL1i with ^{68}Ga. $^{68}GaCl_3$ (444 MBq to 481 MBq) in 0.02 M HCl/98% acetone (800 μL) was transferred to a microcentrifuge tube and heated for 15 min at 95 °C to evaporate the acetone. After adding 200 μL of 0.2 M ammonium acetate buffer (pH 5) to the tube, DOTA-ECL1i was added at a ratio of 1 μg peptide per 4.625 MBq $^{68}GaCl_3$. The reaction

mixture was incubated at 95 °C for 15 min in a thermomixer (600 rpm agitation). The radiolabeled compound was analyzed using radio-HPLC to ensure more than 95% radiochemical purity prior to animal studies.

Serum Stability of ^{68}Ga-DOTA-ECL1i. Purified ^{68}Ga-DOTA-ECL1i (*ca*. 18 MBq) in 1X PBS with 10% mouse serum (Sigma-Aldrich) was incubated at 37 °C in a thermomixer (400 rpm agitation). At each time point (1 h, 2 h, and 4 h), aliquots were filtered through 0.2 μm syringe filter for radio-HPLC analysis of radiochemical purity.

Animal Biodistribution Studies. All animal studies were performed in compliance with guidelines set forth by the NIH Office of Laboratory Animal Welfare and approved by the Washington University Animal Studies Committee. C57BL/6 mice (Charles River Laboratory, Wilmington, MA), Tnnt2-DTR, Tnnt2-DTR/CCR2 KO mice, and mice at four days post ischemia reperfusion injury were used for the biodistribution studies. About 740 kBq of ^{68}Ga-DOTA-ECL1i in 100 μL saline (APP pharmaceuticals, Schaumburg, IL) was injected *via* the tail vein. The mice were anesthetized with inhaled isoflurane for tail vein injections for PET/CT imaging. Inhaled isoflurane was administered prior to euthanasia by cervical dislocation at 1 h post injection, n = 4-8/time point). Organs of interest were collected, weighed, and counted in a Beckman 8000 gamma counter (Beckman, Fullterton, CA). Standards were prepared and measured in parallel with the experimental samples to calculate percentage of the injected dose per gram of tissue (%ID/g).

Generation of Tnnt2-DTR mice

The rat Tnnt2 promotor (-500 bp) was subcloned into pTRECK6. The rat Tnnt2 promotor was a kind gift from Dr. Bin Zhou. pTRECK6 contains a multicloning site followed by the β-globin intron and modified version of the human HB-EGF cDNA harboring I117V and L148V mutations. HB-EGF I117V/L148V lacks capacity to signal via epidermal growth factor receptor but retains sensitivity to Diphtheria toxin (DT). pTRECK6 was a kind gift from Kenji Kohno. Linearized plasmid was injected in C57/CBA blastocysts and transferred into pseudo-pregnant mice. Founders were then identified by PCR and screened by western blot and immunohistochemistry for selective expression of HB-EGF in the heart. Antibodies for western blot were HB-EGF (R&D, cat#AF-259-SP) and GAPDH (Cell Signaling clone: D16H11 cat#5174S). Antibodies for immunohistochemistry include HB-EGF (R&D, cat#AF-259-SP) and cardiac actin (Sigma AC1-20.4.2, cat#A9357). Suitable Tnnt2-DTR founder animals were crossed to C57/B6 mice for 3 generations prior to performing experiments. Cardiomyocyte injury was induced by administering 25 ng DT (Sigma) *via* intraperitoneal injection to Tnnt2-DTR transgenic mice. Cardiomyocyte cell death was examined by intraperitoneal injection of 1% Evans blue diluted in sterile PBS. Evans Blue was administered 24 hours following DT administration. Animals were euthanized 24 hours later, hearts fixed in 2% paraformaldehyde, and Evan's blue uptake examined in 12 μm frozen sections using confocal microscopy.

Ischemia Reperfusion Injury

Two to four month old C57/B6 mice or $CCR2^{GFP/GFP}$ mice on the C57/B6 background (JAX) were anesthetized with sodium pentobarbital, intubated, and mechanically ventilated. A midline incision was made to expose the heart and an 8-0 prolene suture was then 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 exposing the exteriorized suture. 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 abnormality using simultaneous echocardiography. Following 90 minutes of ischemia time, tension was released and the suture ends were placed back into the subcutaneous pocket. The skin was then closed as described above. Sham animals underwent the identical procedure with the exception that tension was not placed on the suture ends. Randomization was employed to select mice for the sham or ischemia procedure. No mice were excluded from the analysis of the ischemia reperfusion experiments. Histology was performed by fixing the heart in 2% paraformaldehyde overnight at 4°C, dehydrating in 70% ethyl alcohol, and embedding in paraffin. Four μm sections were cut and stained with H&E. Mouse echocardiography was performed in the Washington University Mouse Cardiovascular Phenotyping Core facility using the VisualSonics 770 Echocardiography System. Avertin (0.005 ml/g) was used for sedation based on previously established methods of infarct quantification. 2D and M-mode images were obtained in the long and short axis views. Ejection fraction (EF) 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 group. Each experimental group included at least 5 animals.

MicroPET/CT Imaging. At day 4 after cardiomyocyte injury, Tnnt2-DTR mice were anesthetized with isoflurane and injected with 5.55 MBq of ^{68}Ga-DOTA-ECL1i in 100 μL of saline *via* the tail vein. Small animal PET scans (40 to 60 min dynamic scan) were performed on either microPET Focus 220 (Siemens, Malvern, PA) or Inveon PET/CT system (Siemens, Malvern, PA). For mice with ischemia reperfusion injury, same ^{68}Ga-DOTA-ECL1i imaging protocol as Tnnt2-DTR mice was performed at day 1, 4, 7, 14, and 21 after injury. The mice received ^{68}Ga-DOTA-ECL1i scan were fasted overnight prior to the injection of 7.4 MBq ^{18}F-FDG in 100 μL of saline *via* the tail vein. Static scan (10 min) was performed on these mice at 1 h post injection at day 5, 8, 15 and 22 post injury. Polar maps were also generated based on ^{18}F-FDG PET data of the animals to visualize myocardium function. The microPET images were corrected for attenuation, scatter, normalization, and camera dead time and co-registered with microCT images. All of the PET scanners were cross-calibrated periodically. The microPET images were reconstructed with the maximum a posteriori (MAP) algorithm and analyzed by Inveon Research Workplace. The uptake was calculated as the percent injected dose per gram (%ID/g) of tissue in three-dimensional regions of interest (ROIs) without the correction for partial volume effect.

Autoradiography. Autoradiography when combined with PET will provide a more comprehensive and accurate assessment of radiotracer binding to CCR2 expression in the tissue. Typically, following PET imaging, mice are perfused with saline to remove the blood. Hearts are

collected, sliced into multiple pieces with a razor blade, placed on a phosphor-imaging plate for overnight exposure then imaged with a Typhoon FLA 9500 phosphorimager (GE, Marlborough, MA) and quantified using ImageJ. For fixed human heart tissue, sections are deparaffinized and hydrated in PBS and then incubated with ~1 nM ^{68}Ga-DOTA-ECL1i for 15 min at 25 °C. Blocking studies were performed by co-incubation with 100-fold non-radioactive DOTA-ECL1i following the same procedure. Slides were washed 30 times with water then placed on a phosphor-imaging plate for overnight exposure, followed by the detection using a Typhoon FLA 9500 phosphorimager (GE, Marlborough, MA) and quantified using ImageJ. A region of interest was drawn around the entire tissue section. The average pixel intensity of each region of interest was used for the calculation of relative counts ratio.

Human Pathologic Specimens. This study was approved by the Washington University in St. Louis Institutional Review Board (#201305086). All subjects provided informed consent prior to sample collection and the experiments were performed in accordance with the approved study protocol. Cardiac tissue specimens were obtained from adult patients with idiopathic cardiomyopathy (ICM) undergoing left ventricular assist device (LVAD) implantation or cardiac transplantation. Patients with autoimmune disease, active infections, HIV, and hepatitis C were excluded. Tissues consisted of transmural specimens obtained from the apical or lateral wall of the left ventricle. Explanted hearts were flushed by cannulating the left and right coronary artery ostia and perfusing 200 mL of cold saline. Apical cardiac tissue cores resected from patients undergoing LVAD implantation were flushed by cannulating an epicardial vessel and perfusing 50 mL of cold saline. Specimens were then immersed in cold saline and were either immediately flash frozen or fixed in 10% formalin upon collection to preserve tissue integrity.

A second set of human cardiac tissue specimens was obtained from patients who died from acute myocardial infarction (acute coronary event 3-6 days prior to time of death). These samples were provided from the department of pathology of the University Hospital Heidelberg/ tissue bank of the National Center for Tumor Diseases, Heidelberg, Germany in accordance with its regulations and the approval of Heidelberg University's ethics committee.

Immunohistochemistry. Paraffin embedded sections were dewaxed in xylene, rehydrated, endogenous peroxide activity quenched in 10% methanol and 3% hydrogen peroxide, processed for antigen retrieval by boiling in citrate buffer pH 6.0 containing 0.1% Tween-20, blocked in 1% BSA, and stained with the following primary antibodies (with indicated dilution), overnight at 4 °C: CD68 (KP1 eBiosceince, cat#14-0688-82, 1:2000), CCR2 (7A7 Abcam, cat#ab176390, 1:2000), and GFP (Abcam, cat# ab13970, 1:2000). The PerkinElmer Opal Multicolor IHC system was utilized to visualize antibody staining per manufacturer protocol. Immunofluorescence was visualized on a Zeiss confocal microscopy system. Macrophages were quantified by examining at least 4 similarly oriented sections from 4 independent samples, in blinded fashion. Isotype controls and non-transgenic (GFP-) mice were used to validate antibody specificity.

Flow Cytometry. To generate single cell suspensions, saline perfused cardiac tissue specimens were finely minced, and digested in DMEM with Collagenase type 1 (450 U/ml), Hyaluronidase (60 U/ml), and DNase I (60 U/ml) for 1 hour at 37 °C. All enzymes were sourced from Sigma. Digested samples were then filtered through 40 μM cell strainers and washed with cold HBSS that was supplemented with 2% FBS and 0.2% BSA. Red blood cell lysis was performed with

ACK lysis buffer (Thermo Fisher Scientific). Cells were washed with HBSS and resuspended in 100 µL of FACS buffer (DPBS containing 2% FBS and 2 mM EDTA). Cells were then stained with CD45-PercpCy5.5 (30-F11, Biolegend cat# 103131), CD64-PE (X54-5/7.1, Biolegend cat# 139303), Ly6C-APC (HK1.4, Biolegend cat# 128015), CCR2-BV421 (SA203G11, Biolegend cat# 150605), and MHC-II (I-A, I-E) APC/Cy7 (M5/114.15.2, Biolegend cat# 107627) at 4°C for 30 minutes in the dark. All antibodies were sourced from Biolegend. Stained single cell suspensions were washed twice with FACS buffer and resuspended in a 0.35 ml volume. FACS analysis was performed on BD LSR II platform. Isotype control antibodies were used to validate our flow cytometry gating strategy[2].

RT-PCR

RNA was extracted from myocardial tissue using the RNeasy RNA mini kit and Tissue Lyser II (Qiagen). RNA concentration was measured using a nanodrop spectrophotometer (ThermoFisher Scientific). cDNA synthesis was performed using the High Capacity RNA to cDNA synthesis kit (Applied Biosystems). Quantitative real time PCR reactions were prepared with sequence-specific primers (IDT) with PowerUP™ Syber Green Master mix (ThermoFisher Scientific) in a 20 µL volume. Real time PCR was performed using QuantStudio 3 (ThermoFisher Scientific). mRNA expression was normalized to 36B4. Primers were purchased from IDT.

Statistical Methods. Data were analyzed by using software (Prism, version 6.07; GraphPad, La Jolla, Calif). Differences between groups were compared by using nonparametric two-tailed t tests. Means of nonparametric data from human samples were compared with the Mann-Whitney

U test. Multiple means were compared by using a one- or two-way analysis of variance with the Tukey test. $P < 0.05$ was indicative of a statistically significant difference.

References

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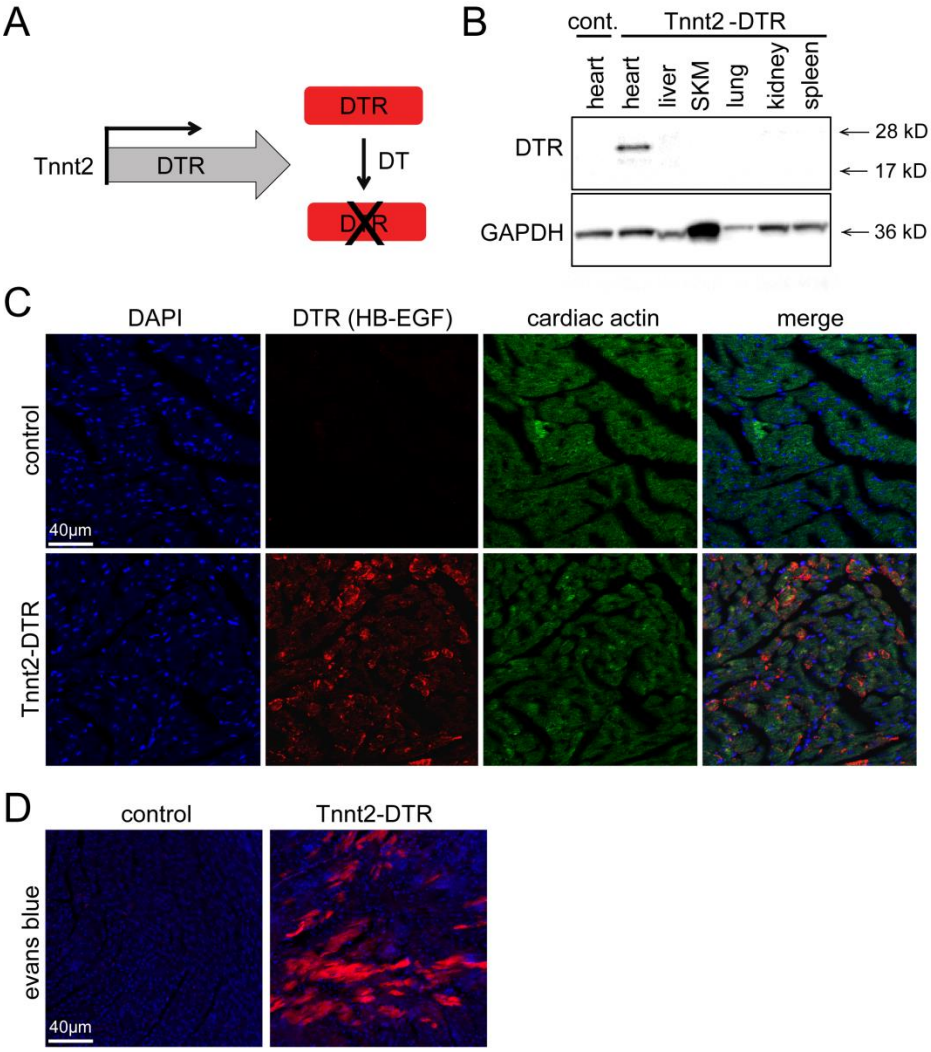
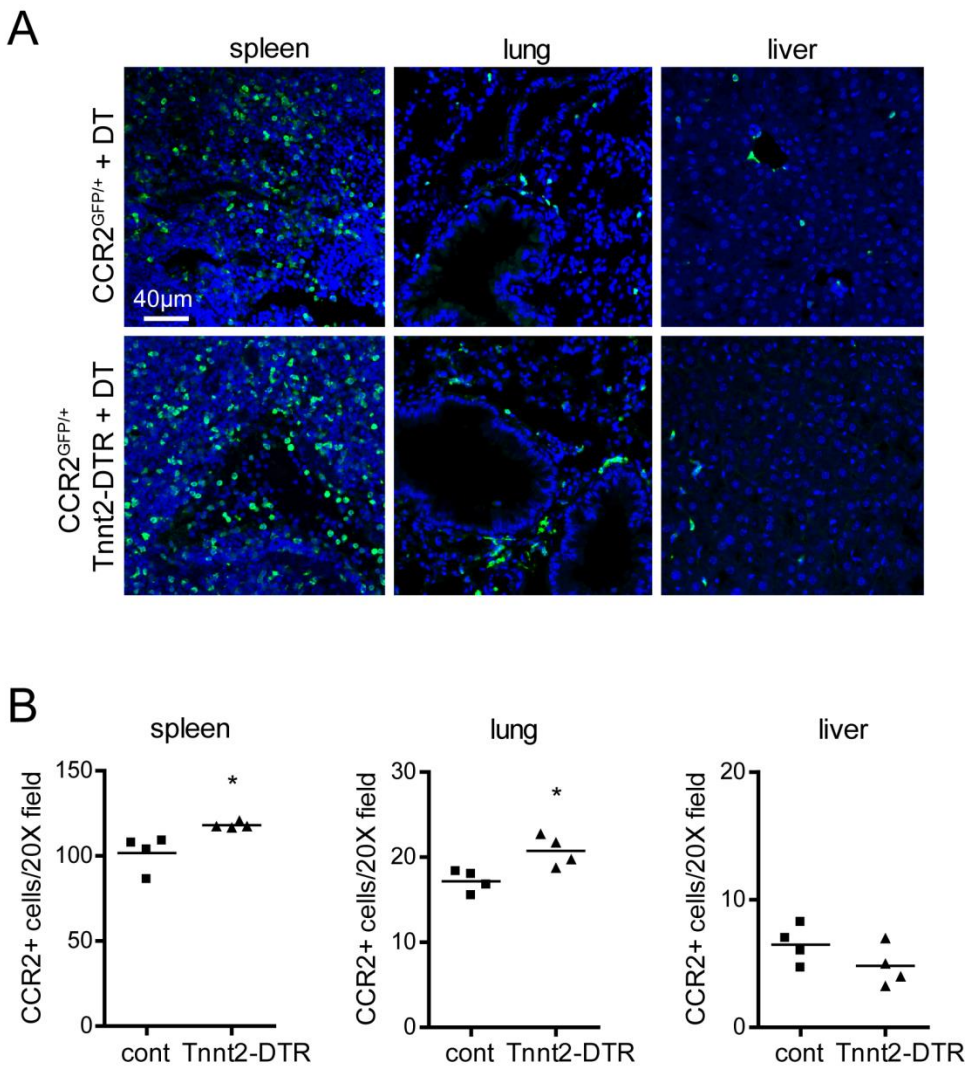
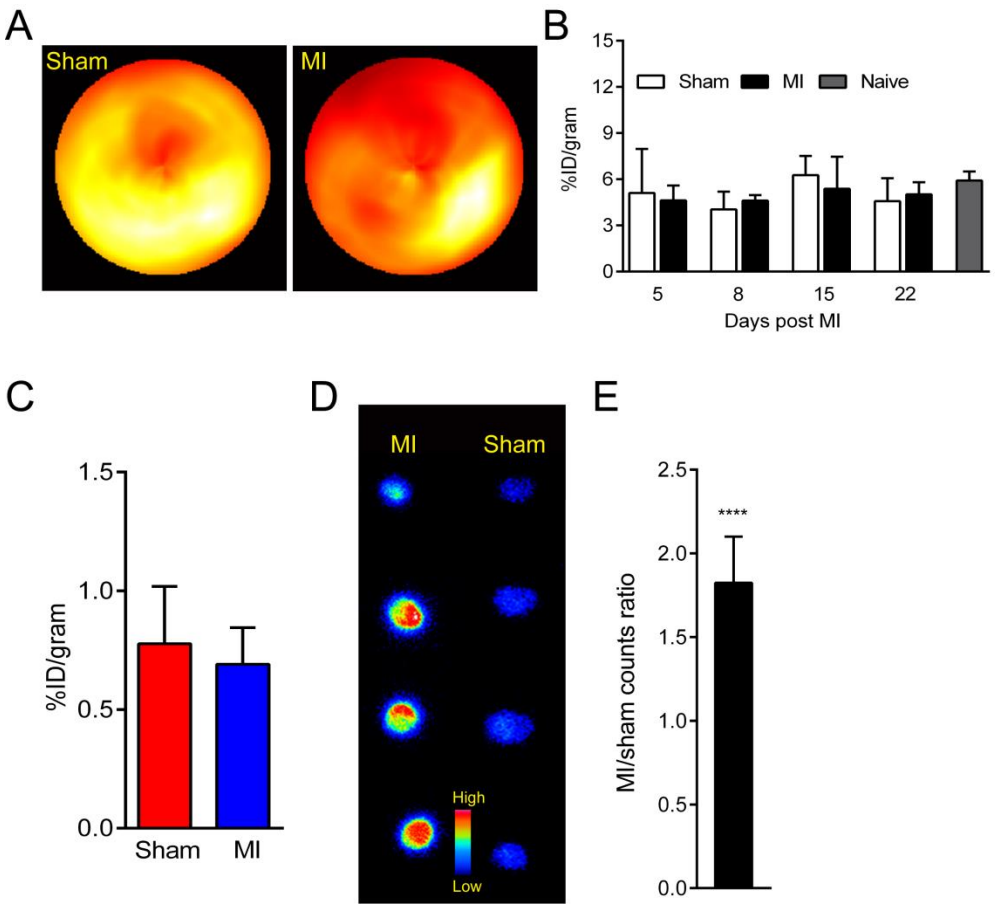


Online Figure I. **(A)** Schematic depicting the strategy utilized to generate Tnnt2-DTR transgentic mice. Tnnt2: Troponin T2 promotor, DTR: Diphtheria toxin receptor, DT: diphtheria toxin. Red rectangles denote cardiomyocytes. **(B)** Western blot for DTR (human HB-EGF) showing selective expression of DTR in the heart of Tnnt2-DTR transgenic mice. **(C)** Immunostaining for DTR (red), cardiac actin (green), and DAPI (blue) showing selective expression of DTR in cardiomyocytes within the hearts of Tnnt2-DTR transgenic mice. 200X magnification. **(D)** Evans blue staining (red) showing evidence of cardiomyocyte cell death in Tnnt2-DTR hearts 48 hours following administration of DT (25 ng IP). Blue: DAPI. SKM: skeletal muscle.



Online Figure II. **(A)** Representative images of GFP immunostaining (green) in the hearts of $CCR2^{GFP/+}$ (control) and Tnnt2-DTR $CCR2^{GFP/+}$ mice 4 days following DT treatment. **(B)** Quantitative analysis of CCR2+ cell abundance in the spleen, lung, and liver of $CCR2^{GFP/+}$ (control) and Tnnt2-DTR $CCR2^{GFP/+}$ mice 4 days following DT treatment. n=5 per experimental group. * $p< 0.05$.



Online Figure III. **(A)** Polar map analysis of ^{18}F-FDG uptake in the hearts of mice after sham manipulation and ischemia-reperfusion injury (labeled MI) mice highlighting the anterior and apical location of the myocardial infarction. **(B)** Quantitative analysis of 18F-FDG PET of naïve, sham, and MI hearts at the indicated time points following ischemia reperfusion injury. Due to small infarct size in this model of closed-chest ischemia reperfusion injury no reduction in total 18F-FDG signal was evident. n=4-5 per experimental group. **(C)** ^{68}Ga-DOTA-ECL1i PET signal intensity in the aortas of sham and MI mice showing minimal blood pool retention. **(D)** Representative autoradiographs of hearts from sham and MI mice following ^{68}Ga-DOTA-ECL1i PET imaging. Analysis was performed 4 days after ischemia-reperfusion injury. **(E)** Quantitative comparison of radioactivity measured from heart autoradiographs of sham and MI mice. n=5 per experimental group. * p< 0.05, ** p< 0.01, *** p< 0.005, **** p<0.001.

Online Table I. Cohort Demographics

Age (yrs)	66.9 (54-77)
Male	81.8
Hypertension	54.5
Hyperlipidemia	81.8
Diabetes	63.6
PVD	36.4
CKD	36.4
Atrial fibrillation	63.6
Infarct age (yrs)	5.1 (0.2-12)
Infarct location	
anterior	54.5
inferior	45.5
lateral	36.4

All values denote percentage of patients unless otherwise noted. Values in parenthesis indicate data range. Yrs: years, PVD: peripheral vascular disease, CKD: chronic kidney disease.