Online Supplemental Materials

Rad GTPase deletion attenuates post-ischemic cardiac dysfunction and remodeling

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Supplemental Methods

MI surgeries and echocardiographic monitoring. Cardiac infarct was generated via surgical ligation of the left anterior descending (LAD) artery as previously described (1). Briefly, mice were anaesthetized with isoflurane, after which a small incision was made in the skin. The heart was carefully manipulated with forceps through the ribcage, where a 6-0 silk suture was tied around the LAD, disrupting flow to the apical myocardium. After replacing the heart to its original position, the wound was sutured and mice were allowed to recover. Cardiac function and dimensions were monitored using m-mode and b-mode echocardiography before surgery, and at 24-hours, 7-days, and 28-days post MI using Vevo2100 software (VisualSonics). For acute infarct measurement, after 24-hours mice were euthanized and hearts were perfused with Evans Blue to determine ischemic area, followed by incubation in 0.1% triphenyl tetrazolium chloride (TTC) to delineate viable myocardium from necrotic tissue.

Acute global ischemia. Mice were anaesthetized with ketamine/xylazine (90/10 mg/kg ip), and hearts were rapidly excised. Retrograde perfusion with an oxygenated low-potassium Tyrode's buffer maintained at 37º was initiated by aortic cannulation. After

clearing of blood and equilibration, flow was halted for 15 minutes, after which hearts were immediately perfused with 0.1% triphenyl tetrazolium chloride (TTC) to identify viable tissue. Hearts were cut by hand into 5-7 sections, and each section was weighed. Viable (red) tissue and necrotic (white) tissue areas were evaluated using ImageJ, and multiplied by the section weight to determine infarct volume.

Cardiomyocyte isolation and calcium transient analysis. Mice were anaesthetized with ketamine/xylazine (90/10 mg/kg ip), and hearts were rapidly excised and cannulated via the aorta. Hearts were perfused with 37º high-potassium Tyrode's buffer containing 2,3-butanedione 2-monoxime (BDM) to arrest contraction until blood was cleared from the heart, after which the heart was perfused with 5-7 mg of liberase (Roche) at 37º. Digestion was followed by physical tissue disruption to separate individual myocytes, which were placed in a 10% FBS solution to inhibit enzyme activity. Calcium was restored to cell preparation in a step-wise fashion to 1mM. All imaging studies were done at room temperature in a 1.8mM calcium physiological salt solution (PSS). Individual myocyte calcium cycling and contraction was measured by loading cells with 2μM Fura-2AM, and field-stimulating cells at 1 Hz at room temperature. Calcium transient peak amplitude, decay constant (tau, τ), and fractional shortening were recorded.

Late gadolinium enhancement (LGE) magnetic resonance imaging (MRI). Infarct size was measured using LGE MRI 1 day after LAD ligation by injecting gadolinium-diethylenetriamine-pentaacetic acid (Gd^{2+}-DPTA 0.1mg/kg) through an intraperitoneal cannula. MRI was performed using a 7-Tesla Bruker ClinScan system (Bruker, Ettlingen, Germany), equipped with a 4-element phased array cardiac coil.

Anaesthetized mice were monitored for breathing rate, temperature, and heart rate via ECG during image acquisition. A cardiac and respiratory gated, multi-slice T1-weighted inversion recovery pulse sequence (2) was acquired for seven ventricular short-axis slices spanning the left ventricle during diastasis. For imaging analysis, the field of view was set to 25.6 x 25.6 mm with a matrix of 128 x 128, a slice thickness of 1 mm, and inversion time of 550 ms. For image analysis, endocardial and epicardial contours and infarct regions (i.e. regions of late gadolinium enhancement) were manually segmented using custom MATLAB software (The MathWorks, Inc., Natick, MA) by a single observer blinded to group designation. Scar burden was quantified as the ratio of infarct volume to total myocardial volume.

Histology. Sham or MI hearts were perfused with phosphate-buffered saline followed by 10% formalin in PBS after 24 hours and 5 weeks. Fixed hearts were halved along the short axis with papillary muscle visible, and both basal and apical regions were sectioned at 5μm increments. In hearts 28 days after surgery, scar and fibrosis development were revealed using Masson's Trichrome staining for collagen deposition in sections from base and apex, which were subsequently averaged to give a final value. To evaluate infiltration of neutrophils into the myocardium, sections from 24-hours post-surgery were de-parafinized, rehydrated, subjected to antigen retrieval, and incubated with polyclonal antibodies for Gr-1. Stained sections were visualized at 20x using Nikon Elements software, and numbers of positively identified neutrophils were averaged over 1 mm^2.

TUNEL staining. Hearts were fixed and sectioned as described above. Apoptotic nuclei were identified using the *In Situ* Cell Death Kit – TMR (Roche). Non-apoptotic nuclei

were identified using DAPI. 10 random, non-overlapping fields were acquired using Nikon Elements software at 20x magnification, and apoptosis was taken as the average number of apoptotic nuclei/total nuclei.

Flow cytometry. 24 hours after surgery, hearts from MI and sham mice were excised and minced and incubated in a mixture of dispase II and collagenase II (Roche) for 30 minutes @ 37◦C in water bath with frequent vortexing. The enzyme activity was terminated by addition of two volumes of ice-cold Flow buffer containing 10% FBS and then particulate material including debris were strained out. The cell suspension was centrifuged for 5min at 450×g and the cell pellet was resuspended in Flow buffer. Cells were incubated with antibodies to CD45-APC-Cy7 (Clone 30-F11), CD115- PE (Clone AFS98), Ly-6G/Ly-6C- PerCPCy5.5 (Clone RB6-8C5) (BioLegend), to distinguish neutrophils from fibroblasts and monocytes.

RNA isolation, microarray, and qRT-PCR. 24 hours after MI or sham surgery, tissue from apical regions of the left ventricle was snap frozen in liquid N_2. Frozen sections were homogenized in Trizol (Invitrogen). RNA was extracted with chloroform followed by isopropanol precipitation and an ethanol wash. The RNA pellet was then treated with DNAse1 (Qiagen) to remove DNA, then precipitated in ammonium acetate and ethanol. After resolubilization, RNA was quantitated and evaluated for quality using Agilent bioanalyzer RNA 6000 RNA nano kit. A Mouse Gene 2.0 ST array was used to probe for gene expression differences between $Rad^{-/-}$ and WT MI hearts. Candidate inflammatory genes identified by this array were validated by RT-PCR using cDNA generated with qScript (VWR) followed by quantitative PCR using Taqman probes for

Il16, *Il1b*, *Cxcl1*, *Cxcl2*, *Cxcr2*, and *Tnf* (Thermofisher). CT values were compared to determine statistical significance between groups.

Statistical analysis. Survival curves were compared using a Wilcoxon-Mann-Whitney test for difference. One-way analysis of variance (ANOVA) was used to determine statistically significant differences between sham and MI WT and $Rad^{-/-}$ groups for single endpoints, followed by post-hoc Student's t-tests for individual comparisons. For longitudinal studies examining multiple time points (i.e. all echocardiograph time courses), a two-way ANOVA was used. A p-value of less than 0.05 was considered significant.

References Cited

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