"SUPPLEMENTAL MATERIAL"

Supplementary Methods

MHC-TRAF2 transgenic mice. The TRAF2 cDNA used for to generate the MHC-TRAF2 construct was the generous gift of Dr. Yongwon Choi.[1] To facilitate separation of endogenous TRAF2 from the TRAF2 that was driven by the alpha-myosin heavy chain promoter, we FLAG tagged the TRAF-2 construct using an EcoRV cleavage site with the primer sequence: CTA GGA TAT CCG TTT AGT GAA CCG TCA GAA TTG ATC TAC CAT GGA CTA CAA AGA CGA TGA CGA CAA GGC TGC AGC CAG TGT GAC. The FLAG-tag was inserted at the N-terminus using a NotI cleavage site with the primer sequence: ACG TGC GGC CGC CTA GAG TCC TGT TAG GTC. The transgene construct were injected into single cell embryos of FVB mice at the Baylor College of Medicine transgenic core facility.

Founder lines of MHC-TRAF2 transgenic mice (FVB background) were identified by PCR and confirmed by Southern blotting using a 286 bp probe targeted to exon 8 of the TRAF2 gene. Western blotting was performed to confirm the expression of TRAF2 protein in the hearts of the MHC-$TRAF_{LC}$ mice using a rabbit anti-TRAF2 (1:5000) Ab that recognizes the N-terminus of TRAF2 (sc-877 Santa Cruz Biotechnology, Santa Cruz, CA) and a anti-FLAG Ab (1:3000) (Sigma-Aldrich, St. Louis, MO) that recognizes the genetically engineered TRAF2 construct.

TRAF2 mediated signaling leads to activation of nuclear factor-kappaB (NF- κB) and c-Jun N-terminal kinase (JNK).[2] Accordingly, we sought to determine whether these pathways were activated in the MHC-$TRAF2_{LC}$ mice using electromobility gel shift assays (EMSAs) to measure NF-κB activation, an in gel kinase assay to measure JNK activity (see data supplement

for details). Briefly, freshly excised hearts from MHC-TRAF2 and littermate control mice were perfused retrograde with cold Krebs Henseleit buffer and snap frozen in liquid nitrogen. Nuclear extracts were obtained using NE-PER Nuclear® and Cytoplasmic Extraction Reagents following the manufacturer's instructions (Pierce, Rockford, IL). EMSAs were performed using NF- κB oligonucleotide consensus sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3' [Santa Cruz Biotechnology, Santa Cruz, CA]), as described.[3] The specificity of the binding was determined by competition with a 20X molar excess of the respective unlabeled oligonucleotide, as well as by supershift assays using polyclonal antibodies directed against the p50 and p65 subunits of NF-κB (sc-109X and sc-114X, respectively, Santa Cruz Biotechnology, Santa Cruz CA). The positive control for this study was a nuclear extract from a littermate control obtained from a mouse that was treated with lipopolysaccharide (LPS) for 1 hour. JNK activity was determined in littermate control and TRAF2 hearts at 4, 8 and 12 weeks of age, using the SAPK/JNK Assay kit (Cat # 9810 from Cell Signaling Technology, Inc., Danvers, MA), according to the manufacturer's instructions. Briefly, c-JUN fusion protein beads were used to capture JNK from tissue extracts, and were probed with a phospho c-Jun (Ser 63) antibody to measure JNK activity. JNK activity was measured at baseline 30 minutes after I/R injury.

Evans blue staining. Because triphenyltetrazolium chloride (TTC) staining may underestimate the true extent of tissue injury within the first 3 hours of cardiac injury,[4] we used Evans blue dye uptake to assess the degree of myocardial tissue injury following ischemia reperfusion injury (see data supplement). Evans blue is a cell impermeable diazo dye that has been used to study the integrity/permeability of blood vessels and cell membranes that become injured. In muscle cells with permeable membranes Evans blue dye crosses into the cell and

accumulates in myofibrils, where it emits red auto-fluorescence when examined using fluorescence microscopy. [5] At the end of the reperfusion protocol (i.e., 60 minutes of reperfusion), the hearts were perfused (1 mL/min) first with 3 mL of ice-cold 0.1% Evans blue dye (Sigma Chemical, St. Louis, MO) diluted in 1x PBS, followed by perfusion with 10 mL of ice-cold 1x PBS, which was used to clear Evans blue dye from the interstitium. The hearts were then subsequently perfused with 10 mL of Z-fix fixative (1 mL/min), paraffin-embedded and sectioned (0.5 μm). To quantify the extent of Evans blue dye uptake, 10 pre-determined radially arranged transmural LV sections were chosen for evaluation in each heart. Each transmural slice through the LV wall was further divided into thirds in order to allow for analysis of the endocardium, the mid-wall, and the epicardium. Fluorescence microscopy (200x) was performed using a filter set with an excitation of 510 - 560 nm and an emission of 590 nm in order to assess the amount of Evans blue dye uptake in the myocardium at baseline and following I/R injury. Hearts were examined at the level of the papillary muscle, using a total of 30 microscopic fields per heart. Data are expressed as the percent area of the myocardium with red fluorescence.

TGF-beta-activated kinase (TAK1) activity. Given that TRAF2 signals through activation of TGF-beta-activated kinase (TAK1),[6] and can that TAK1 can influence cardiac structure and the response to cell injury,[7] we sought to determine whether TAK1 was activated in the MHC-$TRAF2_{LC}$ mice at baseline. TAK1 activity was determined in 10-12 week old naive mouse hearts using an immune complex assay, as described.[8] Briefly, samples were homogenized and endogenous TAK1 was immunoprecipitated using polyclonal antisera against TAK1 (Santa Cruz, California). The precipitates were suspended in a kinase assay buffer (25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM *B*-glycerophosphate, 0.1 mM Na_3VO_4, 10 mM *p*-

nitrophenyl phosphate) containing ATP (20 *u*M), [γ-^{32}P] ATP (5 *u*Ci), and substrate protein His-MKK6 (2 *u*g). The kinase reaction was performed for 30 min at 30˚C and stopped by adding sample buffer. The reaction products were separated by SDS-PAGE and subjected to autoradiography, as described.[8]

Cardiac structure and hemodynamics. We used 2D-targeted M-mode echocardiography and hemodynamic catheterization to characterize LV structure and LV hemodynamics, respectively in littermate control and MHC-TRAF2$_{LC}$ mice (10-12 weeks of age). Isoflurane (1.5% Isoflurane and 98.5% oxygen) was used as anesthesia for all measurements. In a subset of mice that underwent echocardiography, LV end-systolic and end-diastolic pressure were assessed using a Millar 1-Fr micro-tipped pressure catheter (SPR-1000), and Aortic pressure, LV end-diastolic pressure, and peak + LV dp/dT and peak -dp/dT were determined.

Supplementary Results

TAK1 activity. Figure S-1 shows that TAK1 activity was barely detectable, and was similar at baseline in the MHC-TRAF2$_{LC}$ and littermate control mice.

Cardiac structure and hemodynamics. There was a small but significant ($p < 0.02$) difference in mean aortic pressure in the littermate control mice (85 $\pm$ 2.6 mmHg, n = 8) when compared to the MHC-TRAF2$_{LC}$ mice (74.8 $\pm$ 3.5 mmHg, n = 8). As shown in Supplemental Figure S-2, there was no difference in LV end-diastolic dimension, LV mass, LV wall thickness, nor the ration of LV radius to LV wall thickness (r/h ratio) in the littermate and MHC-TRAF2$_{LC}$ mice. There was however, a significant decrease in LV fractional shortening and positive LV dp/dt in the MHC-TRAF2$_{LC}$ mice when compared to wild-type controls ($p < 0.01$). Figure S-3

shows that isovolumetric relaxation (normalized for heart rate) and LV - dp/dT LV were significantly in the MHC-TRAF2$_{LC}$ mice when compared to wild-type controls ($p < 0.05$), whereas indices of LV filling, including, E/A ratio and LV end-diastolic pressure were not significantly ($p > 0.05$) different in the MHC-TRAF2$_{LC}$ and littermate control mice.

Supplementary Figures

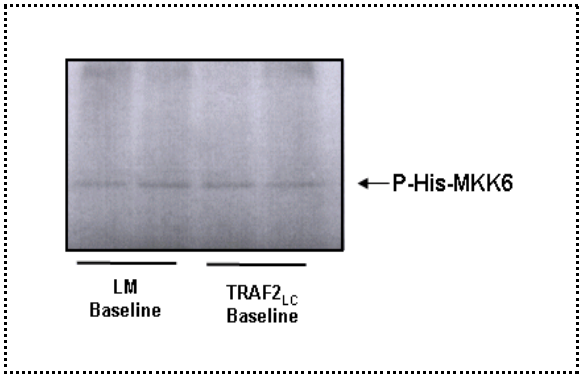

Figure S-1: TGFβ-activated kinase (TAK1) activity in MHC-TRAF2$_{LC}$ mice and littermate controls (naive hearts).detected by an immune kinase assay, The arrow indicates histidine tagged MKK6, which was used as the substrate for TAK1.

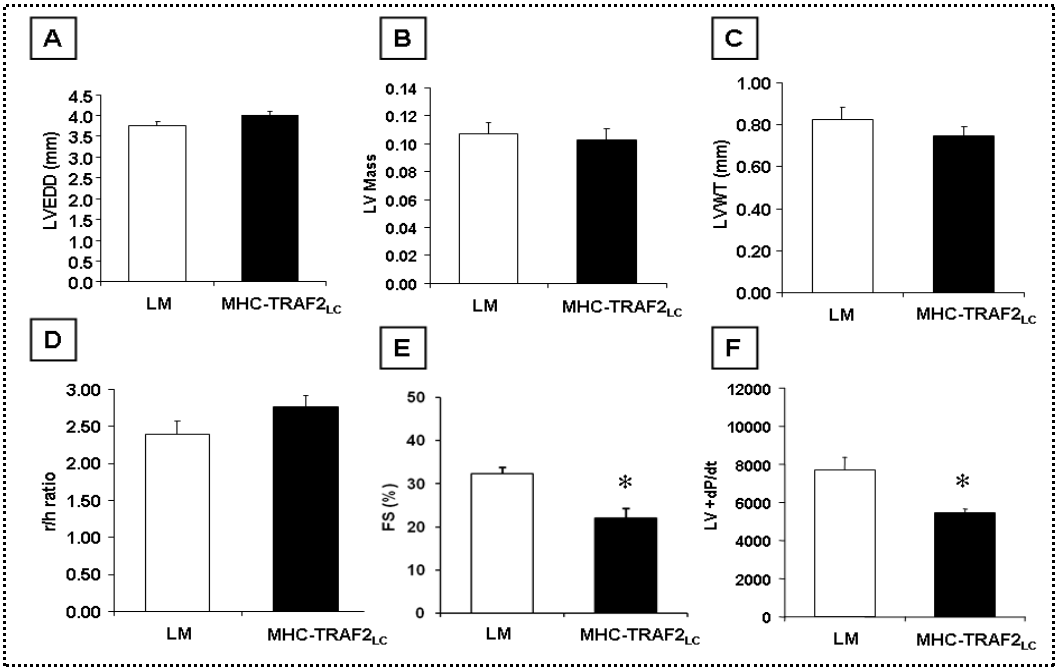

Figure S-2: LV Structure and function in MHC-TRAF$_{LC}$ and littermate (LM) controls . (A) LV dimension (LVEDD), (B) LV mass, (C) LV wall thickness (LVWT), (D) ratio of LV radius to LV wall thickness (r/h ratio), (E) percent LV fractional shortening (% FS) and (F) peak positive LV dp/dt (+ Lvdp/dT) in the MHC-TRAF2$_{LC}$ mice (black bars) compared to LM (open bars). (* = $P < 0.01$ compared to control) (n = 8-10) mice/group for 2D directed echocardiography and n = 8 mice/group for hemodynamic data)

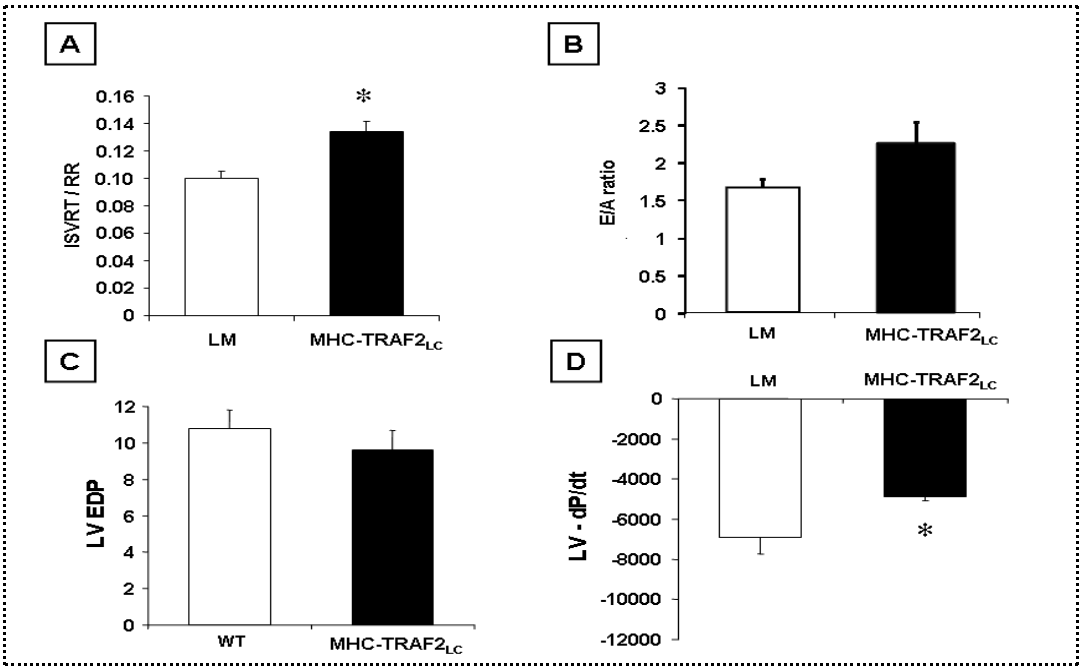

Figure S-3: Diastolic function in MHC-TRAF2$_{LC}$ littermate (LM) controls. (A) LV isovolumic relaxation time normalized by heart rate (ISVRT/RR), (B) ratio of early diastolic (E) to late diastolic (A) filling (E/A ratio), (C) LV end-diastolic pressure (LV edp) and (D) and peak negative LV dp/dt (-dp/dT) was significantly decreased, consistent with impairment of LV diastolic function in the MHC-TRAF2$_{LC}$ mice (black bars) compared to LM controls (open bars).(* = $p < 0.001$ compared to WT) (n = 8- 10 mice/group for 2D directed echocardiography and n = 8 mice/group for hemodynamic data)

Supplementary References

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