Cardiomyocyte specific MLK3 deletion opposes left ventricular dysfunction and eccentric remodeling after pressure overload

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Abstract

Heart failure (HF) is a common syndrome with high mortality, for which there is no cure. Drugs which activate cGMP-dependent protein kinase 1α (PKG1α) have emerged as promising therapies for HF but are frequently limited by hypotension due to vascular relaxation. Thus, there is an interest in understanding downstream substrates of cGMP-dependent protein kinase 1α (PKG1α) in the myocardium which might promote PKG1 therapeutic effects in HF but avoid excess vasodilation. MLK3 functions as a direct substrate effector of PKG1α. Whole-body MLK3 deletion in mice increases cardiac dysfunction and remodeling after pressure overload and prevents the therapeutic effect of cGMP augmentation on LV function. However, MLK3 deletion also causes hypertension, which may confound these findings. We tested the hypothesis that MLK3 opposes LV dysfunction through a blood pressure-independent function in the cardiac myocyte (CM). The objective of this study was to determine the cardiac myocyte (CM) and blood pressure-independent effects of mixed lineage kinase 3 (MLK3) in the regulation of left ventricular (LV) function at baseline and in response to pathological stress. We generated mice harboring LoxP sites flanking the MAP3K11 gene encoding MLK3 (MLK3fl/fl) and crossed them with αMHC-Cre transgenic mice, enabling constitutive postnatal cardiac myocyte-specific MLK3 deletion (CMKO). Twelve-week-old male MLK3 CMKO and control MLK3 intact (MLK3fl/fl Cre-) littermates were studied in the basal state and 14 and 27-28 days after transaortic constriction (TAC) or sham surgery. We assessed LV structure and function by organ mass, echocardiography, in vivo hemodynamics, qPCR, and histological analysis. In basal and TAC studies, we used age-matched αMHC-Cre x MLK3+/+ (non-floxed) mice as additional controls.
MLK3 gene excision in CMs was confirmed by PCR. We observed minimal differences in LV structure and function at baseline between genotypes. After TAC, MLK3 CMKO mice developed a more severe reduction in LV contractile function as assayed by echocardiographic measures of LV ejection fraction and isovolumetric relaxation time; and invasive hemodynamic indices of rate of LV relaxation, maximum LV pressure and LV developed pressure. The MLK3 CMKO LVs also demonstrated a more eccentric remodeling phenotype in which LV mass/tibia length, LV end diastolic and end systolic diameters increased more severely in CMKO mice, with corresponding reductions in LV wall thicknesses. Lung mass/tibia length was elevated in the MLK3 CMKO TAC mice compared to MLK3 intact TAC littermates, indicating overt heart failure and congestion. Myocardial interstitial fibrosis was increased in the CMKO mice after TAC. Finally, CMKO mice had increased pathologic gene expression of ANP, and CTGF compared to MLK3 intact controls. No significant difference in systolic blood pressure or developed pressure was observed in CMKO and MLK3 intact sham groups. Additionally, we found that cardiac myocyte Cre-recombinase promotes modest LV dysfunction after TAC independently of MLK3 deletion.

We conclude that MLK3 in the CM opposes LV dysfunction and eccentric hypertrophy after pressure overload through a blood pressure independent mechanism. These findings have important clinical implications, as drugs which activate PKG1 in heart failure improve outcomes but are limited by excess hypotension. As a myocardial PKG1 substrate which opposes pathological LV remodeling through blood pressure-independent mechanisms, MLK3 represents a potential therapeutic target for heart failure.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>CM</td>
<td>Cardiomyocyte</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>EDPVR</td>
<td>End-diastolic pressure volume relationship</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>ESPVR</td>
<td>End-systolic pressure volume relationship</td>
</tr>
<tr>
<td>FS</td>
<td>Fractional shortening</td>
</tr>
<tr>
<td>HF</td>
<td>Heart failure</td>
</tr>
<tr>
<td>HFP EF</td>
<td>Heart failure with preserved ejection fraction</td>
</tr>
<tr>
<td>HFr EF</td>
<td>Heart failure with reduced ejection fraction</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HW</td>
<td>Heart weight</td>
</tr>
<tr>
<td>IVRT</td>
<td>Isovolumetric relaxation rate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricular</td>
</tr>
<tr>
<td>LVH</td>
<td>Left ventricular hypertrophy</td>
</tr>
<tr>
<td>LZ</td>
<td>Leucine zipper</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>max dP/dt</td>
<td>Rate of ventricular contraction</td>
</tr>
<tr>
<td>min dP/dt</td>
<td>Rate of ventricular relaxation</td>
</tr>
<tr>
<td>MKK</td>
<td>Mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>MLK3</td>
<td>Mixed lineage kinase 3</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PKG1</td>
<td>cGMP-dependent protein kinase 1</td>
</tr>
<tr>
<td>PRSW</td>
<td>Preload-recruitable stroke work</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricular</td>
</tr>
<tr>
<td>TAC</td>
<td>Transaortic constriction</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with 0.1% Tween 20</td>
</tr>
<tr>
<td>TL</td>
<td>Tibia length</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Heart failure

Heart failure (HF) is a complex syndrome caused by the inability of the heart to maintain a normal cardiac output required to meet the body’s metabolic needs. HF is the leading cause of morbidity and mortality globally as well as the leading cause of hospitalization in the United States. HF most commonly occurs when left ventricular (LV) structure or function becomes altered leading to impaired ventricular filling or ejection, a process discussed below, termed cardiac remodeling. HF can be further classified by the degree of systolic dysfunction as measured by LV ejection fraction (EF). The two major classifications are HF with reduced EF (HFrEF) with LVEF ≤ 40% and HF with preserved EF (HFpEF) with LVEF ≥ 50%\textsuperscript{1,2}. HFrEF is often preceded by cardiomyocyte loss or dysfunction due to ischemia, genetic mutation, or valvular disease which results in systolic dysfunction. HFpEF is often correlated with risk factors including hypertension, diabetes mellitus, obesity, and renal insufficiency which results in hypertrophy, fibrosis, and inflammation\textsuperscript{3}. Patients differ by classification in prognosis and response to treatment. Mortality is higher in HFrEF than in HFpEF, however few effective treatments for HFpEF have been identified. Despite availability of treatments for HFrEF including diuretics, angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, and β-blockers, mortality remains high\textsuperscript{4}.

1.2 Left ventricular hypertrophy in heart failure

LV remodeling describes molecular and cellular changes that alter LV structure, size, or function. LV hypertrophy (LVH) is a type of remodeling characterized by increased LV mass and enlarged cardiomyocytes (CM). LVH occurs as a means to maintain normal cardiac output in response to increased workload. However, LVH in
response to pathological stimuli ultimately promotes progression of HF by adversely affecting function\(^5\). Hypertrophy due to pressure overload is usually initially characterized by increased ventricular wall thickness without chamber enlargement and is termed concentric hypertrophy. By contrast, eccentric hypertrophy is characterized by chamber dilation with decreased or unchanged ventricular wall thickness\(^6\). Eccentric hypertrophy typically occurs during volume overload. Whole organ hypertrophy is usually associated with similar changes at the cardiac myocyte level. Concentric hypertrophy usually occurs when sarcomeres are added in parallel, resulting in increased myocyte width. By contrast, eccentric hypertrophy occurs when sarcomeres assemble in series resulting in an increase in myocyte length. These two types of hypertrophy may exist as a continuum where both CM length and width are increased\(^5\).

1.3 PKG1 molecular signaling in left ventricular hypertrophy

The presence of LVH predicts cardiovascular mortality and contributes to the pathogenesis of HF\(^7\). Understanding the molecular signaling pathways in the myocardium that contribute to these processes could reveal novel therapeutic targets in HF. The cGMP molecular signaling pathway has been shown to oppose LV remodeling. Augmentation of cGMP by inhibition of phosphodiesterase (PDE) 5 or PDE9 reduces hypertrophy and improves LV function in experimental models of HF\(^8,9\). cGMP interacts with several substrates but cGMP-dependent protein kinase 1 (PKG1) is its main cardiovascular target. cGMP directly activates PKG1 kinase function. PKG1 is required for normal LV function after pressure overload\(^10\), and hormone stimulus\(^11\). Mice with genetic disruption of the PKG1\(\alpha\) isoform-specific leucine zipper (LZ) protein interaction domain experience high mortality, LV dysfunction, and increased HF after pressure overload. Furthermore, the PKG1\(\alpha\) leucine zipper domain is required for the cardiac
hypertrophy attenuating and therapeutic effects of sildenafil. The discovery of the role
PKG1 in cardiac function is fairly recent, however, whereas PKG1 effects on vascular
smooth muscle cell activity have been well established. Vascular PKG1 lowers blood
pressure through vasorelaxation. Furthermore, genetic disruption of the PKG1α LZ
domain leads to vascular smooth muscle cell dysfunction and hypertension in mice.
Drugs that activate PKG1 show improved outcomes in HFrEF, however they are limited
by PKG1-induced vasodilation. Signaling molecules downstream of PKG1 that mediate
therapeutic effects on LV function without promoting blood pressure effects represent
potential therapeutic targets for HF. Several PKG1α substrates have been identified
including cardiac myosin binding protein c, and cardiac troponin I. The existence of
CM-specific PKG1α substrates indicates independent roles of PKG1α in cardiac and
vascular systems, specifically the mechanisms modulating LV function and blood
pressure. However, the aforementioned proteins are difficult to target with drugs and
identification of novel PKG1α signaling substrates is necessary.

1.4 MLK3 in heart failure

Mixed lineage kinase 3 (MLK3) was recently identified as a direct substrate
effector of PKG1α in the myocardium. The MLK family was originally identified as a
serine/threonine kinase with structural homology to tyrosine kinases, leading to the
terminology of mixed lineage. Upon binding with the small GTPase cdc42, MLK3
phosphorylates mitogen activated protein kinase kinase-4 (MKK4) and MKK7 and
stimulates c-Jun N-terminal kinase (JNK) activation. JNK represents one of three
main signaling branches of the mitogen-activated protein kinase (MAPK) family and has
been demonstrated to oppose concentric LVH. MAPK signaling cascades that regulate
a wide variety of cellular processes including cell growth, differentiation, apoptosis, and
transformation, and have been implicated as mediators of cardiac hypertrophy\textsuperscript{22}. MLK3 also inhibits the activation of the GTPase RhoA through an allosteric, kinase independent mechanism\textsuperscript{23}.

MLK3 is required for preservation of LV structure and function after pressure overload. Genetic loss of function studies have revealed an anti-remodeling function of MLK3 in vivo. Whole body MLK3 deletion (MLK3 KO) exhibited increased hypertrophy at baseline and larger LV chamber dimensions, increased LV mass and worsening LV systolic function after pressure overload compared to wild type littermates\textsuperscript{17}. Moreover, MLK3 is required for the therapeutic effects of PKG1 on LV function. In a further genetic loss of function study, PKG1 activator sildenafil improved pulmonary congestion and LV function in wild type mice but not in MLK3 KO mice after induced pressure overload. Unexpectedly, MLK3 KO mice also displayed elevated blood pressure compared to wild type littermates indicating a role of MLK3 in blood pressure modulation. In the same study, sildenafil promoted hypotension in both MLK3 KO and wild type mice, indicating that MLK3 is not required for, and does not mediate PKG1 induced blood pressure reduction. Additionally, inhibition of RhoA normalized blood pressure in MLK3 KO mice supporting that MLK3 normally represses increases in blood pressure through the kinase independent inhibition of RhoA. Finally, pharmacological inhibition of MLK3 in wild type mice did not increase blood pressure indicating that MLK3 inhibition of RhoA occurs through mechanisms independent of MLK3 kinase function\textsuperscript{24}. Therefore, the effect of MLK3 on blood pressure occurs independently of the blood pressure lowering effect of PKG1α.

Association of Cdc42 with MLK3 requires the MLK3 CRIB domain\textsuperscript{19}. Recent work has identified that the MLK3 CRIB domain is necessary for MLK3 regulation of cardiovascular function. In a study using mice with point mutations that disrupt the MLK3 CRIB domain, the mutant mice exhibited concentric hypertrophy and LV dysfunction at
baseline, compared to wildtype littermates. Similarly to the MLK3 KO mice, disruption of the MLK3 CRIB domain also induced elevated blood pressure. The CRIB mutation caused reduced MLK3 expression in the LV and prevented MLK3 translocation from cytosol to plasma membrane. These effects may have contributed to the observed hypertension by limiting MLK3 access to RhoA. The CRIB domain was also shown to be necessary for MLK3 preservation of LV function after pressure overload. After induced pressure overload, both mutant and wildtype mice developed hypertrophy, however LV function declined selectively in the MLK3 CRIB mutant mice25.

In both the MLK3 KO and MLK3 CRIB mutant models described, MLK3 disruption caused increased hypertrophy and LV dysfunction after pressure overload. However, both models also exhibited pre-existing systemic hypertension that could predispose the heart to damage, contributing to these outcomes. Thus, it remains unclear whether the accelerated LV dysfunction observed after TAC in these mice represents a primary effect of MLK3 deletion from the heart, or rather a secondary effect of chronic hypertension. Additionally, MLK3 is widely expressed throughout the body including in CMs26, vascular smooth muscle cells27, leukocytes28, and fibroblasts29. Therefore, the specific cell type(s) in which MLK3 modulates cardiac response to pressure overload remains unknown. MLK3 inhibition in isolated CMs does lead to cellular hypertrophy in vitro17, suggesting a direct role of MLK3 in the CM in opposing hypertrophy. However, the specific CM-specific role of MLK3 in the basal state or in response to cardiovascular stress has not been examined. The described models do not investigate the cell-type-specific effects of MLK3 in vivo, and therefore, cannot definitively conclude that the observed left ventricular physiological effects occurred independently of MLK3 disruption in other cell types.

We hypothesized that MLK3 opposes LV dysfunction after pressure overload through a blood pressure independent role in the CM. To test this hypothesis, we
generated mice harboring LoxP sites flanking the MAP3K11 gene encoding MLK3 (MLK3fl/fl) and crossed them with αMHC-Cre transgenic mice, enabling constitutive postnatal cardiac myocyte-specific MLK3 deletion (CMKO). We examined the baseline and LV pressure overload-induced cardiovascular effects of the MLK3 CM deletion compared to MLK3 intact littermates. We also used age-matched αMHC-Cre x MLK3+/+ (non-floxed) mice as additional controls.
Chapter 2: Methods and Materials

2.1 Study approval

All mouse care and investigational protocols were approved by the Tufts University School of Medicine and Tufts Medical Center's Institutional Animal Care and Use Committee, protocol B2021-97.

2.2 Experimental animals

MLK3 cardiomyocyte-specific deletion (CMKO) mice and MLK3 intact littermates were obtained by breeding mice harboring LoxP sites flanking the MAP3K11 gene encoding MLK3 (MLK3fl/fl) with αMHC-Cre transgenic mice in the animal facility at Tufts Medical Center. MLK3fl/fl mice were generated in partnership with Ingenious Targeting Laboratory. αMHC-Cre mice were obtained from The Jackson Laboratory. Non-floxed controls were obtained by breeding αMHC-Cre with wildtype MLK3+/+ mice. Lines were maintained on a C57BL/6 background. Male mice between 11 and 14 weeks of age were used in this study. Investigators were blinded to animal genotype prior to surgeries and subsequent analysis.

2.3 Echocardiography

Cardiac function in 11-14-week-old mice was evaluated by echocardiography prior to surgery, then at 14 and 27 days following surgery. For the baseline study, echocardiography was performed 1-4 days before surgery. Echocardiography was performed as previously described using the Vevo 2100 ultrasound system and 40-MHz transducer (Visualsonics, Toronto, Canada.) Mice were anesthetized using 2.5% isoflurane in oxygen at 1L/min, then maintained under 1% isoflurane in the supine
position on an electrical heating pad set to 39°C. A heat lamp was also used to maintain core temperature. ECG monitoring was obtained using limb electrodes. Hair in the thoracic region was removed using depilatory cream (Nair). Scanning was performed in parasternal long and short axis views in B-Mode and M-Mode. LV structural analysis was performed in parasternal short axis using M-Mode imaging and measurements were used to calculate ejection fraction and fractional shortening. Transvalvular flow velocity profiles were measured in apical four-chamber view using color and tissue doppler images. Mitral inflow velocity and isovolumetric relaxation time was acquired by pulse-wave doppler, and septal annular e’ velocity was acquired by tissue doppler. Image depth, width, and gain settings were adjusted to optimize image quality.

2.4 Surgery
MLK3fl/fl CMKO mice and intact littermates, along with non-floxed Cre+ and Cre-littermates aged 11-14 weeks, were randomly assigned to either sham surgery or transaortic constriction (TAC) surgery. TAC was performed as previously described with a 26-gauge needle to tighten the suture at the transverse aorta between the two carotid arteries. Mice were anesthetized with 2.5% gaseous isoflurane and maintained on a small animal ventilator during surgery.

2.5 Hemodynamic analysis
LV ventricular function was measured 28 days following surgery through in vivo pressure-volume analysis as previously described. Mice were anesthetized with 3% isoflurane and maintained at 2% isoflurane using a small animal ventilator. Core temperature was maintained at 37°C using a rectal thermometer-lamp system. A pressure-volume transducing catheter was introduced into the right carotid artery, then
placed into the LV by crossing the aortic valve. Hemodynamic data were recorded and analyzed using IOX software (EMKA v. 2.5.1.10).

2.6 Tissue isolation
Following hemodynamic analysis, the heart and lungs were removed, and the tibia length was measured. The heart was separated into four chambers and bathed in isotonic saline. The lungs and chambers were weighed separately, followed by snap-freezing in liquid nitrogen, and then stored in -80°C. The mid LV in the short axis orientation was separated and stored in 10% neutral buffered formalin.

2.7 Histological analysis
The mid LV was fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4µm sections using a microtome. Sections were stained for collagen with picrosirius red to visualize fibrosis. Sections were imaged using an Olympus trinocular microscope at a 40x objective and SPOT imaging software (v. 5.6). Analysis was conducted using the NIH ImageJ software. Percentage of total fibrosis area was determined by quantifying the percentage of stained area in each image. Perivascular and interstitial fibrosis were quantified by manually fitting ellipses around vessels and adjusting their size using scaling factors of 1.50 for both the x and y axes. The percentage of perivascular fibrosis was calculated by quantifying the stained area within the ellipse selection, while the percentage of interstitial fibrosis was calculated by quantifying the stained area outside of the ellipse selection. Five images from each sample were analyzed and the average stained areas are reported.
2.8 Quantitative RT-PCR

Tissue from the base of the LV was homogenized on dry ice by pulverization using a stainless steel mortar and pestle. The resulting powder was weighed and 1mL TRIzol was added/100mg tissue to extract total LV RNA. RNA was isolated using Direct-zol RNA Miniprep Plus kit (Zynmo Research), followed by reverse transcription of 2 µg RNA to cDNA using QuantiTect Reverse Transcriptase kit (Qiagen). Target primers and cDNA samples were incubated in a 96 well plate in duplicate and amplified by quantitative real-time PCR (qRT-PCR) using SsoFast EvaGreen Supermix (Bio-Rad) according to manufacturer’s recommendations. Primers for qRT-PCR analysis were as follows: Gapdh Fwd 5’- AGG TCG GTG TGA ACG GAT TTG -3’, Rev 5’- TGT AGA CCA TGT AGT GGT CA -3’; Nppa Fwd 5’- TCG TCT TGG CCT TTT GGC T -3’, Rev 5’- TCC AGG TGG TCT AGC AGG TTC T -3’; Nppb Fwd 5’- AGG TCC TAG CCA GTC TCC AGA -3’, Rev 5’- GAG CTG TCT CTG GGC CAT TTC -3’; Ctgf Fwd 5’- GGG CCT CCT CTG CGA TTT C -3’, Rev 5’- ATC CAG GCA AGT GCA TTG GTA -3’. All samples were amplified for 40 cycles performed at 95°C for 5s and 55°C for 30s using the QuantStudio 3 Real-Time System (ThermoFisher Scientific). qPCR data were analyzed using the ΔΔCt method with Gapdh as the reference control, and values were normalized to fold change.

2.8 Immunoblotting

LV tissue was homogenized on dry ice by pulverization using a stainless steel mortar and pestle, as described above. The resulting powder was lysed with 200µL tissue lysis buffer consisting of 20mM HEPES, 50mM β-Glycerol Phosphate, 2mM/L EGTA, 1mM/L DTT, 10 mM/L NaF, 1mM/L NaVO₄, 1% Triton-X 100, and 10% Glycerol per 10mg. The mixture was vortexed 20s/sample and kept on ice for 1hr while agitating samples every
20min before centrifugation and the resulting supernatant was saved. Protein concentrations were quantified by Bicinchoninic Acid (BCA) Assay using the Micro BCA Protein Assay Kit (ThermoFisher Scientific). LV tissue lysates were diluted 1:1 with 2x Laemmli Sample Buffer containing SDS (Sigma S-3401) and vortexed. The mixture was boiled at 100°C to denature proteins. Protein samples and protein ladder marker (Bio-Rad Precision Plus Dual Color Standards) were loaded into 8% polyacrylamide gels and separated by SDS PAGE running buffer (ChemCruz; 10X running buffer) for approximately 1 hr. Proteins were then transferred to nitrocellulose membranes (Bio-Rad, 1620094) in transfer buffer (10x TGS 10%, distilled Water 70%, Methanol 20%). Ponceau S was used to ensure successful transfer before blocking with 5% non-fat milk powder in in tris-buffered saline with 0.1% Tween 20 (TBST). The membrane was then treated with the primary antibody overnight in 4°C. Primary antibodies used included anti-MLK3 (1:500 diluted in 5% Bovine Serum albumin (BSA)/TBST; Abcam Scientific, Ab51068) and Anti-GAPDH (EMD Millipore, MAB374) (1:1,000 diluted in 5% BSA/TBST; Cell Signaling Technology). Membranes were then treated with secondary ECL Anti-rabbit IgG Horseradish peroxidase-linked whole antibody (1:2000 diluted in 5% non-fat milk powder in in TBST; GE Healthcare, NA934) for 1hr at room temperature. Membranes were washed briefly in TBST, and then imaged using the chemiluminescent substrate (ThermoFisher Scientific, 34095) and ProteinSimple FluorChem E system.

2.9 PCR
CMs were isolated from female MLK3 CMKO or MLK3 intact mice as described17. DNA was isolated from CMs or from spleen tissue from the same mice by Qiagen DNeasy kit. PCR was performed with the following conditions; proteins were denatured at 94°C for 2 minutes, followed by amplification of samples for 30 cycles performed at 94°C for 30s,
58°C for 30s, and 72°C for 60s. Fragments were separated by gel electrophoresis (1% agarose) with ethidium bromide, and imaged by ultraviolet light. We used the following primers: F1, 5'-TCAGGTTCTCTTTGAAAGCAGAGCCAC - 3'; R1, 5'-GGCTGCAGCAACAGAACTGGG -3'; R2, 5'-CCTGCCTCTGGATCAGCTCAACTC -3'.

2.10 Statistical analysis
All data are presented as mean ± SEM. Comparisons between two groups were performed with Student’s two-tailed unpaired t test. For analysis of multiple groups, we used 2-way ANOVA with Šidák's multiple comparisons test for multiple comparisons as indicated in figure legends. Values of P ≤ 0.05 were considered statistically significant.

2.11 Contributions
All surgeries were performed by Gregory Martin. The immunoblot and PCR experiments to confirm CM-specific MLK3 deletion in MLK3 CMKO mice were performed by Santo Kalathingal Anto. Picrosirius red staining was performed by members of the Tufts Histology Core. Study planning, animal husbandry, echocardiography, hemodynamic analysis, tissue isolation, histological analysis, qRT-PCR, and all data analysis were performed by Anna Burke.
Chapter 3: Results

3.1 Confirmation of CM-specific MLK3 deletion in MLK3 CMKO mice

We generated mice harboring LoxP sites flanking the MAP3K11 gene encoding MLK3 (MLK3fl/fl) and crossed them with αMHC-Cre transgenic mice, enabling constitutive postnatal cardiac myocyte-specific MLK3 deletion (Fig. 3.1A). We confirmed CM specific deletion in the MLK3fl/fl/αMHC-Cre+ (CMKO) mice compared to MLK3fl/fl/αMHC-Cre- (MLK3 Intact) littermates and MLK3 global deletion mice (MLK3 KO) by immunoblot (Fig. 3.1C) and PCR (Fig. 3.1C, D).

Figure 3.1 Selective MLK3 gene excision in cardiac myocyte.
A) Schematic of full-length and excised MLK3 gene showing Forward (F1) primer (covering LoxP sequence) and Reverse (R1, R2) primers to confirm excision. L: loxP sites. B) Predicted PCR fragment sizes. C) Immunoblot for MLK3 and GAPDH in LV tissue from MLK3fl/fl/αMHC-Cre- (MLK3 Intact), MLK3fl/fl/αMHC-Cre+ littermates (MLK3 CMKO), or MLK3 global deletion mice. Representative of 3 separate experiments. D) PCR on DNA from isolated cardiac myocytes or spleen of MLK3fl/fl/αMHC-Cre- or MLK3fl/fl/αMHC-Cre+ littermates. Blue arrow: predicted F1-R2 band in intact gene; Red arrow: 1 kb product from F1-R2 in excised gene; Black arrow: predicted 0.5 kb F1-R1 product in intact gene.
3.2 Minimal phenotypic differences in LV structure and function in MLK3 fl/fl and non-floxed Mice at baseline

To examine the baseline effects of MLK3 deletion from the CM, we assessed LV structure and function by echocardiography in MLK3 fl/fl and non-floxed mice of both Cre+ and Cre- genotypes, 2 days prior to TAC or sham surgery (Table 3.1). At baseline, LV structure was not significantly changed in MLK3 fl/fl, Cre+ (denoted CMKO) mice compared with MLK3 fl/fl Cre- (denoted MLK3 intact) littermates, or in Cre+ non-floxed mice compared with Cre- littermates. Isovolumetric relaxation rate (IVRT) was decreased in CMKO mice compared to intact littermates. The mitral valve E/e’ ratio was decreased in Cre+ non-floxed mice compared to Cre- littermates. Otherwise, no basal differences in LV function were observed within each group.

Table 3.1 Baseline LV structure and function in MLK3 fl/fl and non-floxed mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MLK3 fl/fl Mice</th>
<th>MLK3 No-flox Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cre- (MLK3 Intact), n = 20-21</td>
<td>Cre- (MLK3 No-flox, n = 10</td>
</tr>
<tr>
<td>Septal wall thickness, mm</td>
<td>0.80 ± 0.03</td>
<td>0.87 ± 0.05</td>
</tr>
<tr>
<td>Posterior wall thickness, mm</td>
<td>0.87 ± 0.04</td>
<td>0.91 ± 0.06</td>
</tr>
<tr>
<td>End diastolic diameter, mm</td>
<td>3.76 ± 0.09</td>
<td>0.96 ± 0.05</td>
</tr>
<tr>
<td>End systolic diameter, mm</td>
<td>2.63 ± 0.09</td>
<td>3.24 ± 0.12</td>
</tr>
<tr>
<td>FS, %</td>
<td>30.24 ± 1.27</td>
<td>6.96 ± 0.05</td>
</tr>
<tr>
<td>EF, %</td>
<td>57.93 ± 1.84</td>
<td>60.66 ± 2.83</td>
</tr>
<tr>
<td>IVRT, ms</td>
<td>20.70 ± 1.12</td>
<td>17.23 ± 0.90</td>
</tr>
<tr>
<td>Mitral inflow E, mm/s</td>
<td>613.57 ± 27.24</td>
<td>631.55 ± 27.64</td>
</tr>
<tr>
<td>Mitral inflow A, mm/s</td>
<td>473.57 ± 17.26</td>
<td>487.47 ± 19.39</td>
</tr>
<tr>
<td>E/A</td>
<td>1.30 ± 0.05</td>
<td>1.30 ± 0.04</td>
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<tr>
<td>Septal e’, mm/s</td>
<td>-16.58 ± 1.18</td>
<td>-17.93 ± 2.39</td>
</tr>
<tr>
<td>E/e’</td>
<td>-39.03 ± 2.22</td>
<td>-42.14 ± 4.22</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>448.81 ± 11.52</td>
<td>475.73 ± 13.50</td>
</tr>
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</table>
Echocardiographic analysis was conducted in 11–14-week-old MLK3 fl/fl and non-floxed mice prior to transaortic constriction (TAC) or sham treatment. Data were analyzed using two-tailed unpaired t-test and expressed as means ± SEM. FS, fractional shortening; EF, ejection fraction; IVRT, isovolumetric relaxation time; HR, heart rate.

3.3 Reduced LV function and accelerated dilation in MLK3 CMKO mice at 14 days post-TAC

To test the effects of MLK3 CM deletion in the setting of LV pressure overload, we next performed 26-gauge TAC or sham surgery on the above mice. We evaluated LV structural and functional parameters by echocardiography 14 days following surgery (Table 3.2). Compared to MLK3 intact littermates, MLK3 CMKO mice had increased end systolic and diastolic diameters as well as decreased ejection fraction after 14 days of TAC (Fig. 3.2A). LV structure did not significantly differ in Cre+ non-floxed mice compared to Cre- littermates. However, ejection fraction decreased in Cre+ non-floxed mice compared with Cre- littermates after 14 days of TAC (Fig. 3.2B).

<table>
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<tr>
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<th>Cre+ fl/fl</th>
<th>Cre+ non-floxed</th>
<th>p-value</th>
</tr>
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<tr>
<td>End diastolic diameter, mm</td>
<td>3.51 ± 0.12</td>
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<tr>
<td>End systolic diameter, mm</td>
<td>2.15 ± 0.35</td>
<td>2.11 ± 0.41</td>
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</tr>
<tr>
<td>FS, %</td>
<td>38.89 ± 2.52</td>
<td>39.16 ± 1.86</td>
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<tr>
<td>EF, %</td>
<td>69.59 ± 3.16</td>
<td>70.18 ± 2.36</td>
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</tr>
<tr>
<td>IVRT ms</td>
<td>15.85 ± 1.17</td>
<td>16.21 ± 1.50</td>
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<tr>
<td>Mitral inflow E, mm/s</td>
<td>695.90 ± 37.59</td>
<td>700.18 ± 24.20</td>
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</tr>
<tr>
<td>Mitral inflow A, mm/s</td>
<td>536.75 ± 24.64</td>
<td>533.71 ± 33.82</td>
<td>ns</td>
</tr>
<tr>
<td>E/A</td>
<td>1.30 ± 0.05</td>
<td>1.37 ± 0.09</td>
<td>ns</td>
</tr>
<tr>
<td>Septal e', mm/s</td>
<td>-21.38 ± 5.28</td>
<td>-15.64 ± 4.94</td>
<td>0.0193</td>
</tr>
<tr>
<td>E/e'</td>
<td>-31.17 ± 1.76</td>
<td>-48.58 ± 1.43</td>
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<tr>
<td>HR, bpm</td>
<td>500.22 ± 14.30</td>
<td>473.17 ± 13.04</td>
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Echocardiographic analysis was conducted in 11–14-week-old MLK3 fl/fl and non-floxed mice prior to transaortic constriction (TAC) or sham treatment. Data were analyzed using two-tailed unpaired t-test and expressed as means ± SEM. FS, fractional shortening; EF, ejection fraction; IVRT, isovolumetric relaxation time; HR, heart rate.
Echocardiographic analysis was conducted in MLK3 fl/fl and non-floxed mice 14 days following 26-gauge transaortic constriction (TAC) or sham treatment. Data was analyzed using 2-way ANOVA with Šidák’s multiple comparisons test and expressed as means ± SEM. FS, fractional shortening; EF, ejection fraction; HR, heart rate.

<table>
<thead>
<tr>
<th>Table 3.2 Cardiac structure and function in MLK3 fl/fl and non-floxed mice subjected to TAC for 14 days.</th>
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<th>MLK3 fl/fl Mice</th>
<th>Septal wall thickness, mm</th>
<th>Posterior wall thickness, mm</th>
<th>End diastolic diameter, mm</th>
<th>End systolic diameter, mm</th>
<th>FS, %</th>
<th>EF, %</th>
<th>HR, bpm</th>
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<td>Sham</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cre−, n = 9</td>
<td>0.84 ± 0.05</td>
<td>0.86 ± 0.06</td>
<td>3.81 ± 0.10</td>
<td>2.73 ± 0.16</td>
<td>28.81 ± 2.35</td>
<td>55.56 ± 3.57</td>
<td>479 ± 14.48</td>
</tr>
<tr>
<td>Cre+, n = 4</td>
<td>0.79 ± 0.02</td>
<td>0.81 ± 0.05</td>
<td>4.17 ± 0.20</td>
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<td>31.95 ± 4.57</td>
<td>59.61 ± 6.17</td>
<td>427 ± 32.93</td>
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</tr>
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</tr>
<tr>
<td>Cre−, n = 8</td>
<td>1.00 ± 0.04</td>
<td>0.98 ± 0.04</td>
<td>4.08 ± 0.11</td>
<td>3.07 ± 0.19</td>
<td>25.29 ± 2.88</td>
<td>49.59 ± 5.07</td>
<td>478.75 ± 31.14</td>
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<td>Cre+, n = 5</td>
<td>1.09 ± 0.12</td>
<td>0.88 ± 0.07</td>
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<td>4.11 ± 0.33</td>
<td>12.17 ± 2.21</td>
<td>26.05 ± 4.48</td>
<td>551 ± 70.3</td>
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<td>0.0065</td>
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<th>Septal wall thickness, mm</th>
<th>Posterior wall thickness, mm</th>
<th>End diastolic diameter, mm</th>
<th>End systolic diameter, mm</th>
<th>FS, %</th>
<th>EF, %</th>
<th>HR, bpm</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cre−, n = 5</td>
<td>0.94 ± 0.06</td>
<td>0.97 ± 0.08</td>
<td>3.60 ± 0.10</td>
<td>2.26 ± 0.12</td>
<td>37.40 ± 1.82</td>
<td>68.19 ± 2.45</td>
<td>443 ± 22.93</td>
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<tr>
<td>Cre+, n = 5</td>
<td>1.12 ± 0.03</td>
<td>1.03 ± 0.11</td>
<td>3.56 ± 0.25</td>
<td>2.13 ± 0.22</td>
<td>40.77 ± 2.66</td>
<td>72.14 ± 3.09</td>
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</tr>
<tr>
<td>Cre−, n = 5</td>
<td>1.31 ± 0.05</td>
<td>1.23 ± 0.12</td>
<td>3.56 ± 0.17</td>
<td>2.30 ± 0.22</td>
<td>35.93 ± 3.97</td>
<td>65.60 ± 5.23</td>
<td>466.4 ± 29.99</td>
</tr>
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<td>Cre+, n = 3</td>
<td>1.27 ± 0.06</td>
<td>1.34 ± 0.16</td>
<td>3.51 ± 0.26</td>
<td>2.62 ± 0.10</td>
<td>24.97 ± 2.51</td>
<td>50.22 ± 3.83</td>
<td>456.33 ± 27.33</td>
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Figure 3.2 MLK3 CM-specific deletion leads to increased LV dilation and reduced function at 14 days post-TAC
Left ventricular (LV) parameters measured by echocardiography in (A) MLK3 fl/fl and (B) non-floxed mice 14 days following 26-gauge transaortic constriction (TAC) or sham surgery. Data was analyzed using 2-way ANOVA with Šidák's multiple comparisons test. ns, P ≥ 0.05; *, P < 0.05; **, P ≤ 0.01.

3.4 Increased eccentric remodeling in MLK3 CMKO mice after 27 days of TAC
After 27 days of TAC or sham surgery, we again evaluated LV structure and function by echocardiography (Table 3.3). After 27 days of TAC, LV septal and posterior wall thickness increased selectively in MLK3 intact mice compared to CMKO mice. End diastolic and systolic diameters increased significantly in CMKO mice compared to MLK3 intact littermates. These findings indicate increased eccentric remodeling in MLK3 CMKO mice after pressure overload (Fig. 3.3A, C). In non-floxed mice, LV end systolic diameter did increase as a function of TAC, indicating induction of LV systolic dysfunction in these groups. However, we observed no significant differences in wall
thickness, end diastolic diameter, or end systolic diameter between genotypes within surgery groups (Fig. 3.3B).

Figure 3.3 MLK3 CM-specific deletion leads to increased eccentric LV remodeling at 27 days post-TAC.
Left ventricular (LV) structural parameters measured by echocardiography in (A) MLK3 fl/fl and (B) non-floxed mice 27 days following 26-gauge transaortic constriction (TAC) or sham surgery. Data was analyzed using 2-way ANOVA with Šídák's multiple comparisons test. ns, P ≥ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001. (C) Representative M-Mode images of the LV in MLK3 fl/fl mice 27 days following TAC or sham surgery.

3.5 Reduced LV function in MLK3 CMKO mice after 27 days of TAC
We evaluated LV function after 27 days of TAC or sham surgery using ejection fraction and IVRT measurements from echocardiography (Table 3.3). In MLK3 fl/fl mice, ejection fraction decreased in both TAC groups, with a significantly greater decrease observed in the MLK3 CMKO mice compared to MLK3 intact littermates. IVRT increased in CMKO
mice compared to intact littermates after TAC (Fig. 3.4A), consistent with diastolic dysfunction. We observed a similar pattern in the non-floxed mice, in which ejection fraction decreased and IVRT increased after 27 days of TAC in the Cre+ mice compared to Cre- littermates (Fig. 3.4B). However, this occurred to a lesser extent than in the MLK3 fl/fl group (Fig. 3.4C).

Figure 3.4 MLK3 CM-specific deletion leads to reduced LV function at 27 days post-TAC.
Ejection fraction and isovolumetric relaxation time (IVRT) measured by echocardiography in (A) MLK3 fl/fl and (B) non-floxed mice 27 days following 26-gauge

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transaortic constriction (TAC) or sham surgery. Data was analyzed using 2-way ANOVA with Šidák's multiple comparisons test. ns, $P \geq 0.05$; *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

3.6 Reduced LV hemodynamic function in MLK3 CMKO mice at 28 days post-TAC

We next evaluated LV function in the same mice by invasive hemodynamics 28 days following TAC or sham surgery (Table 3.3). Systolic blood pressure and developed pressure increased in MLK3 intact mice after 28 days of TAC. By contrast blood pressure in CMKO TAC mice was significantly reduced compared with MLK3 intact mice, consistent with reduced cardiac output. We observed no significant differences in blood pressure between CMKO and MLK3 intact mice after sham surgery. The maximal rate of ventricular relaxation (min $dP/dt$) was significantly decreased in CMKO mice after TAC compared to MLK3 intact littermates. We observed trends towards decreased rate of ventricular contraction (max $dP/dt$) in CMKO compared to MLK3 intact littermates after TAC (Fig. 3.5A). Systolic blood pressure and developed pressure increased to the same degree in both Cre+ and Cre- non-floxed mice after TAC compared to sham but did not significantly differ by genotype. Cre+ non-floxed mice had decreased min $dP/dt$ and trended towards decreased max $dP/dt$ compared to Cre- littermates in both surgery groups (Fig. 3.5B).
Figure 3.5 MLK3 CM-specific deletion leads to reduced left ventricular (LV) hemodynamic function at 28 days post-TAC.
Systolic blood pressure, developed pressure, maximal rate of LV contraction (max dP/dt), and minimal rate of LV relaxation (min dP/dt) evaluated by in vivo hemodynamics in (A) MLK3 fl/fl and (B) non-floxed mice 28 days following 26-gauge transaortic constriction (TAC) or sham surgery. Data was analyzed using 2-way ANOVA with Šídák's multiple comparisons test. ns, P ≥ 0.05; *, P < 0.05; ****, P ≤ 0.0001.

3.7 Increased LV congestion in MLK3 CMKO mice at 28 days post-TAC

Organs were collected and weighed 28 days following TAC or sham surgery (Table 3.3).

In the MLK3 fl/fl mice, TAC induced LV and right ventricular (RV) hypertrophy, compared with sham, indicating the expected phenotype of pressure overload-induced cardiac hypertrophy. RV and lung mass increased in CMKO mice after TAC compared with MLK3 intact littermates (Fig. 3.6A). Non-floxed TAC groups also exhibited LV hypertrophy compared to sham. However, LV, RV, and lung mass did not differ in non-floxed mice by genotype (Fig. 3.6B).
3.6 MLK3 CM-specific deletion leads to increased LV congestion at 28 days post-TAC.

Left ventricle (LV), right ventricle (RV), total heart (HW), and lung masses in (A) MLK3 fl/fl and (B) non-floxed mice 28 days following 26-gauge transaortic constriction (TAC) or sham surgery. Organ masses were normalized to tibia length (TL). Data was analyzed using 2-way ANOVA with Šidák's multiple comparisons test. ns, P ≥ 0.05; *, P < 0.05.

3.8 Direct comparison of MLK3 fl/fl and non-floxed TAC groups

To further investigate the effect of Cre on cardiac structure and function, we performed a subsequent analysis directly comparing MLK3 fl/fl and non-floxed mice after TAC. Cre+ MLK3 fl/fl and non-floxed mice experienced decreased ejection fraction compared to Cre- mice after 14 and 27 days of TAC. Ejection fraction in MLK3 fl/fl Cre+ mice decreased significantly more than in non-floxed Cre+ mice at both 14 and 27 days of TAC (Fig. 3.7A, B). RV and lung mass trended toward an increase in the Cre+ MLK3 fl/fl mice after 28 days of TAC compared with the other TAC groups (Fig. 3.7C). Systolic blood pressure, developed blood pressure and max dP/dt decreased in both Cre+ MLK3 fl/fl and non-floxed mice after 28 days of TAC compared to Cre- mice after TAC. However, this decrease was more pronounced in the Cre+ MLK3 fl/fl group compared to the Cre+ non-floxed group (Fig. 3.7D). These results indicate that the transgenic Cre
protein induced effects on LV function, but that MLK3 deletion induced additive negative effects after TAC independent of Cre alone.

**Figure 3.7 Direct comparison of MLK3 fl/fl and non-floxed TAC mice.**
Ejection fraction measured by echocardiography in MLK3 fl/fl and non-floxed mice at (A) 14 days and (B) 27 days following 26-gauge transaortic constriction (TAC). (C) Right ventricle (RV) and lung masses normalized to tibia length (TL) in MLK3 fl/fl and non-
floxed mice 28 days following TAC. (D) Systolic blood pressure, developed pressure, and maximal rate of LV contraction (max dP/dt) in MLK3 fl/fl and non-floxed mice 28 days following TAC. Data was analyzed using 2-way ANOVA with Šidák’s multiple comparisons test. ns, P ≥ 0.05; *, P < 0.05.

Table 3.3 Echocardiography, hemodynamic, and organ weight data in MLK3 fl/fl and non-floxed mice 27-28 days following TAC or sham surgery

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<tr>
<th>MLK3 fl/fl Mice</th>
<th>Echocardiography</th>
<th>Hemodynamics</th>
</tr>
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<td></td>
<td>Septal wall thickness, mm</td>
<td>Posterior wall thickness, mm</td>
</tr>
<tr>
<td>Sham</td>
<td>Cre-, n = 9</td>
<td>Cre+, n = 6</td>
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<tr>
<td>0.97 ± 0.06</td>
<td>0.88 ± 0.06</td>
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</tr>
<tr>
<td>0.98 ± 0.06</td>
<td>0.95 ± 0.07</td>
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<tr>
<td>3.54 ± 0.16</td>
<td>3.84 ± 0.19</td>
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<td>2.25 ± 0.18</td>
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</tr>
<tr>
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</tr>
<tr>
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Organ Weights

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<td>1.23 ± 0.06</td>
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<td>&lt;0.0001 ns</td>
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<td>Lung/TL, mg/cm</td>
<td>7.64 ± 0.19</td>
<td>7.51 ± 0.15</td>
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<td>BW, g</td>
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**MLK3 No-flox Mice**

**Echocardiography**

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<tr>
<td><strong>Sham</strong></td>
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<td><strong>Cre+, n = 5</strong></td>
<td>P Sham</td>
<td><strong>Cre-, n = 5</strong></td>
<td><strong>Cre+, n = 3-5</strong></td>
<td>P TAC</td>
<td>P Genotype</td>
<td>P Surgery</td>
<td>P Interaction</td>
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<td>Septal wall thickness, mm</td>
<td>1.05 ± 0.08</td>
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<td>ns</td>
<td>1.15 ± 0.08</td>
<td>1.18 ± 0.12</td>
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<td>Posterior wall thickness, mm</td>
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<td>1.08 ± 0.07</td>
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<td>End diastolic diameter, mm</td>
<td>3.44 ± 0.11</td>
<td>3.89 ± 0.16</td>
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<td>3.81 ± 0.12</td>
<td>3.92 ± 0.24</td>
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<td>End systolic diameter, mm</td>
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<td>2.56 ± 0.11</td>
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<td>2.77 ± 0.22</td>
<td>3.25 ± 0.26</td>
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<td>0.0024 ns</td>
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<td>FS, %</td>
<td>38.58 ± 3.82</td>
<td>34.18 ± 1.61</td>
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<td>27.91 ± 3.70</td>
<td>17.53 ± 1.65</td>
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<td>0.0213</td>
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<td>36.96 ± 3.27</td>
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<tr>
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<td>920.05 ± 96.86</td>
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<td>Mitral inflow A, mm/s</td>
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<td>547.78 ± 53.15</td>
<td>ns</td>
<td>584.73 ± 41.28</td>
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<tr>
<td>E/A</td>
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<tr>
<td>Septal e', mm/s</td>
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## Hemodynamics

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<td>End Diastolic Pressure, mmHg</td>
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<td>ns</td>
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<td>Developed Pressure, mmHg</td>
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<td>88.5 ± 2.53</td>
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<td>125.25</td>
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<td>Maximum dP/dt, mmHg/s</td>
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<td>Minimum dP/dt, mmHg/s</td>
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<td>Stroke volume, µL</td>
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<td>15.75</td>
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<td>Contractility index, 1/s</td>
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<td>ns</td>
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<td>± 9.54</td>
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## Organ Weights

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Echocardiographic analysis was conducted in MLK3 fl/fl and non-floxed mice 27 days following 26-gauge transaortic constriction (TAC) or sham treatment. Hemodynamic analysis was conducted 28 days post-TAC. Organ mass was measured 28 days post-TAC and normalized to tibia length (TL). Data was analyzed using 2-way ANOVA with Šidák’s multiple comparisons test and expressed as means ± SEM.

<table>
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<td>28.86 ± 1.50</td>
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<td>5.79 ± 0.20</td>
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<td>9.09 ± 0.54</td>
<td>1.44 ± 0.10</td>
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<td>8.19 ± 0.38</td>
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3.9 Pathological gene expression pattern in MLK3 CMKO mice after 28 days of TAC

Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are fetal genes typically expressed at low levels in the adult heart but can become reactivated in response to pathological hypertrophy. The RNA expression of ANP increased in MLK3 CMKO mice compared to MLK3 intact littermates after TAC. Expression of BNP increased in both CMKO and MLK3 intact mice after TAC compared to sham groups, but genotype did not significantly affect expression (Fig. 3.8A). Connective tissue growth factor (CTGF) promotes cardiac fibrosis and serves as a marker of cardiac dysfunction. RNA expression of CTGF was also increased in CMKO mice over MLK3 intact littermates after TAC (Fig. 3.8B).
Figure 3.8 MLK3 CM-specific deletion leads to a pathologic gene expression pattern at 28 days post-TAC.
RNA expression of (A) atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) and (B) connective tissue growth factor (CTGF) by quantitative PCR in left ventricle (LV) tissues. Data was analyzed using 2-way ANOVA with Šidák’s multiple comparisons test. ns, $P \geq 0.05$; *, $P < 0.05$; ****, $P \leq 0.0001$.

3.10 Increased myocardial interstitial fibrosis in MLK3 CMKO mice after 28 days of TAC
Cardiac fibrosis occurs in response to pathological stress, and often accompanies pathological hypertrophy. We performed picrosirius red staining to visualize collagen in LV sections and identified increased myocardial interstitial fibrosis in MLK3 CMKO mice compared to MLK3 intact littermates after TAC. Additionally, perivascular fibrosis increased in both MLK3 fl/fl TAC groups compared to sham but was not significantly
affected by genotype (Fig. 3.9A). We observed no significant genotypic differences or effects from surgery in fibrosis in the non-floxed mice (Fig. 3.9B).

**Figure 3.9 MLK3 CM-specific deletion leads to increased myocardial interstitial fibrosis at 28 days post-TAC.**
Quantification of total fibrosis, perivascular fibrosis, and interstitial fibrosis in picrosirius red stained left ventricle (LV) tissue sections from (A) MLK3 fl/fl and (B) non-floxed mice 28 days following 26-gauge transaortic constriction (TAC) or sham treatment. Data was analyzed using 2-way ANOVA with Šidák's multiple comparisons test. ns, P ≥ 0.05; ***, P < 0.001.
P ≤ 0.001. (C) Representative images of picrosirius red staining of collagen in LV tissue sections at 40x magnification in MLK3 fl/fl mice 28 days following TAC or sham treatment.
Chapter 4: Discussion

The present study tested the hypothesis that MLK3 opposes LV dysfunction after pressure overload through a blood pressure independent role in the CM. We tested this hypothesis by investigating the effect of CM-specific deletion of MLK3 on cardiac structure and function in the baseline state and in response to LV pressure overload. We observed: 1) no differences in LV hypertrophy after TAC between CMKO and MLK3 intact littermates; 2) increased LV dysfunction in CMKO mice after TAC; 3) increased eccentric hypertrophy in CMKO after TAC; 4) modest effects of transgenic Cre recombinase expression on LV function that were additive to the effects from MLK3 deletion alone. Taken together, these findings support a role of MLK3 in the CM that opposes LV dysfunction and eccentric hypertrophy after pressure overload.

4.1 CM-specific MLK3 deletion induced LV eccentric hypertrophy after TAC

We observed no LV structural effects of Cre expression in both the MLK fl/fl and non-floxed groups at baseline (Table 3.1). However, by 14 days of TAC LV end systolic and diastolic diameter of CMKO mice began to increase compared to MLK3 intact littermates, indicating LV eccentric hypertrophy (Fig. 3.2A). This eccentric hypertrophy became more severe after 27 days of TAC. LV wall thickness increased in MLK3 intact mice after TAC, as expected. However, in the CMKO mice, LV chamber dimension increased more drastically than in the intact mice. Further, LV wall thickness failed to increase in CMKO mice after TAC (Fig. 3.3A, C). We did not observe any similar structural changes in the non-floxed mice after TAC beyond the expected TAC-induced hypertrophy. After 28 days of TAC, both MLK3fl/fl and non-floxed mice showed comparable degrees of LV hypertrophy as evaluated by LV and whole heart weight (Fig.
Taken together, we interpret these findings to indicate that the CMKO TAC mice developed a more eccentric remodeling phenotype compared to MLK3 intact TAC mice. The more severe eccentric remodeling in MLK3 CMKO TAC mice is consistent with observations in MLK3 global deletion mice. For example, in a 2019 study, global MLK3 deletion (MLK3 KO) led to increased LV chamber size after TAC compared with wildtype TAC littermates. Although wall thickness increased in each genotype after TAC, MLK3 KO TAC mice displayed less thick LV walls compared with wildtype TAC mice. However, unlike the CMKO TAC mice, MLK3 global deletion mice displayed increased LV hypertrophy compared with TAC controls. Further, whereas the global MLK3 KO TAC mice had an increase in LV wall thickness after TAC, the MLK3 CMKO mice failed to increase wall thickness at all after TAC (Fig. 3.3A). Several factors could explain this subtle difference. First, the MLK3 effect on LV wall thickness in pressure overload may arise primarily through its function in the CM. Additionally, the current study used a more severe constriction (26g) to induce pressure overload, whereas the global MLK3 KO study used a moderate (25g) TAC. Finally, in the global MLK3 KO study, the MLK3 KO mice showed increased LV hypertrophy at baseline, which could be due to the accompanying hypertension in these mice. Thus, while they displayed increased hypertrophy after TAC compared with wild type TAC, their relative increase in LVH was comparable. In the present study we did not investigate organ weights until 28 days post-surgery so increased basal LV weight in CMKO mice is formally possible. However, this seems unlikely based on the lack of other structural abnormalities observed in our echocardiographic analysis. We interpret our findings to support that the increased LV chamber diameter and eccentric remodeling observed in global MLK3 KO mice likely occurs due to the effect of MLK3 deletion in the CM.

In a later 2022 study, disruption of MLK3 activation through CRIB domain point mutations led to increased LV hypertrophy at baseline compared to wildtype controls but
was not significantly different from wildtype after 1 week of 25-gauge TAC. Basal hypertrophy was determined by increases in LV, RV, and total heart weight as well as echocardiographic measurements of LV wall thickness. This study found that MLK3 disruption resulted in LV wall thickness increases consistent with concentric hypertrophy. In the present study, we did not observe significant echocardiographic differences in LV hypertrophy at baseline which could be due to differences in MLK3 disruption models. In the 2022 study, MLK3 disruption was achieved through point mutations in the CRIB domain and was not cell-type specific. In the present study we used Cre-lox recombination to delete MLK3 specifically in the CM. Additionally, in the 2022 study, LV concentric hypertrophy developed after TAC but did not differ significantly between mutant and wildtype mice\textsuperscript{25}. The present study observed LV structural changes consistent with eccentric hypertrophy after TAC in CMKO mice but not in MLK3 intact littermates. This could be attributed again to differences in animal models but also several differences in methodology. In the present study we used a more severe TAC model and took echocardiographic measurements at later time points. Both studies discussed demonstrate LV hypertrophy after global MLK3 disruption. The current study investigated the effects MLK3 deletion specifically in the CM and found that CM-specific MLK3 plays a role in preventing eccentric hypertrophy after pressure overload.

The molecular mechanisms which cause the switch from concentric to eccentric hypertrophy in the CMKO mice remain unclear. Several signaling pathways have been identified which can induce the different patterns of these two hypertrophy phenotypes. For example, the ERK1/2 MAPK signaling branch promotes concentric hypertrophy. In a 2000 study, transgenic mice over expressing MEK1, a selective kinase for ERK1/2 phosphorylation, showed ERK1/2 hyper-activation and developed concentric hypertrophy\textsuperscript{32}. Eccentric hypertrophy is induced by the MEK5-ERK5 MAPK branch. Mice overexpressing an ERK5 mutant develop ventricular chamber dilation and wall thinning
consistent with eccentric hypertrophy. A third type of hypertrophy distinct from concentric or eccentric hypertrophy is mediated by calcineurin signaling. Calcineurin is activated by elevations in intracellular calcium and binds to its primary downstream effector, nuclear factor of activated T cells (NFAT). Upon binding, calcineurin dephosphorylates NFAT, which enables NFAT translocation to the nucleus where it promotes a pathologic pattern of pro-hypertrophic gene expression. Activation of the calcineurin-NFAT pathway produces dramatic hypertrophy where CMs are disorganized and nearly double in size. Future studies will investigate the contributions of these signaling pathways to the MLK3 effect on hypertrophic patterning.

4.2 CM-specific MLK3 deletion induced LV dysfunction after TAC

In addition to LV structural effects described above, we observed a CM-specific MLK3 effect on LV function after pressure overload. We observed minimal baseline differences between CMKO and MLK3 intact littermates. Only isovolumetric relaxation time (IVRT) was mildly decreased in CMKO mice potentially indicating a slight effect of CM-specific MLK3 on baseline LV diastolic function (Table 3.1). However, other baseline echocardiographic indices of LV diastolic function including E/A and E/e' ratios did not differ between genotypes. Further, our invasive hemodynamic assessment of sham mice revealed no alterations in diastolic parameters (dP/dt min, tau, EDPVR). We therefore conclude that MLK3 CM specific deletion has minimal effects on baseline diastolic function. After 14 days of TAC, however, ejection fraction (EF) decreased significantly in CMKO mice compared to MLK3 intact littermates (Fig. 3.2A). By 27 days post-TAC, EF was even further reduced in CMKO mice indicating severe LV dysfunction. Additionally, IVRT increased more dramatically in CMKO mice indicating LV diastolic dysfunction (Fig. 3.4A). Invasive hemodynamic analysis 28 days after TAC showed a corresponding
reduction in maximum rate of ventricular relaxation (min dP/dt) as well as a trend
towards decreased maximum rate of ventricular contraction (max dP/dt) suggesting
impaired LV diastolic and systolic function respectively (Fig. 3.5A). Furthermore, post-
TAC hemodynamic analysis also showed increased systolic blood pressure and
developed pressure in MLK3 intact but not in CMKO mice despite increased resistance
from TAC, suggesting a decreased cardiac output from MLK3 CM-deletion. Taken
together, we interpret these findings to indicate a requirement of MLK3 in the CM for
maintenance of LV function after pressure overload.

Prior studies have supported that MLK3 disruption promotes LV dysfunction in
pressure overload. In the 2019 study described above, MLK3 KO mice experienced
reduced LV functional abnormalities compared to wildtype littermates after 4 weeks of
TAC but not at baseline. EF was significantly decreased in MLK3 KO mice compared to
littermates after TAC and max dP/dt was significantly decreased in MLK3 KO mice after
TAC compared to after sham\textsuperscript{17}. Additionally, LV function, as evaluated by contractility
index and EF, declined selectively in the MLK3 CRIB mutant mice compared to wildtype
littermates after 1 week of TAC\textsuperscript{25}. Both previous studies discussed involve global
disruption of MLK3 while the current study investigates the role of CM-specific MLK3 on
LV function. We found that CM-specific MLK3 is required for normal LV function after
pressure overload. Our current study therefore identifies the CM as a critical cell type
through which MLK3 maintains LV function after pressure overload.

4.3 CM Cre-recombinase independently promotes LV dysfunction after TAC.

As an additional experimental control, we assessed the effect of the Cre
recombinase alone on the LV response to TAC. We specifically compared Cre+ to Cre-
littermates on a wildtype MLK3 background (i.e. non-floxed MLK3). These mice have
intact MLK3 even in the presence of Cre, so any differential effects observed in Cre+ compared with Cre- can be attributed to Cre toxicity, rather than MLK3 deletion. We observed a modest impact of Cre on LV function at 14 and 27 days of TAC (Fig. 3.2B, Fig. 3.4B). Additionally, after 27 days of TAC, IVRT was increased in Cre+ non-floxed mice compared to Cre- non-floxed mice, indicating more severe diastolic dysfunction. We also note a mild genotypic effect of Cre on min dP/dt (Fig. 3.5B).

These observed effects of Cre on cardiac function have been described in prior literature. In a recent 2023 study, αMHC-Cre mice showed normal cardiac function until around 6 months old when they began to develop arrhythmias, and cardiomyopathy. The αMHC-Cre mice began to die at 6 months old and reached 100% lethality within a year while wildtype littermates maintained 100% survival for at least another 400 days. After 6 months, the αMHC-Cre had developed severe cardiac fibrosis primarily in the left ventricle as well as atrial tumor-like blockages, and reduction of the myocardial intercalated disc. Li et al. found that activation of the ferroptosis signaling pathway played a role in Cre-mediated cardiotoxicity. In an earlier study, transgenic lines expressing Cre recombinase in the heart were generated. Seven transgenic lines developed dilated cardiomyopathy and congestive heart failure. Transgenic lines that expressed low levels of Cre recombinase remained healthy. Consistent with the present study, both previous studies expressed Cre recombinase in the heart using the αMHC promoter. The lines used in the 2006 study were generated by Buerger et al. The 2023 study used B6.FVB-Tg (Myh6-cre) 2182Mds/J founders whereas the present study maintained lines on a C57BL/6 background.

The Cre-lox system is used extensively in cardiac genetic loss of function studies and unexpected Cre effects could confound the effects of the gene of interest. Here we report a mild effect of Cre-recombinase on LV dysfunction after TAC induced pressure overload. Importantly, however, direct comparison of MLK3 fl/fl and non-floxed TAC
groups shows a greater additive effect on LV function from MLK3 deletion than what is observed in the presence of Cre alone (Fig. 3.7A-D). We therefore conclude that while the Cre may have affected LV function, CM-specific MLK3 deletion produces an independent negative effect after TAC.

4.4 CM-specific MLK3 deletion results in pulmonary congestion after TAC

In congestive HF, reduced LV function leads to elevated filling pressures, causing congestion of the pulmonary circulation. This results in fluid leaking into the interstitial space of the lungs. Right ventricular hypertrophy (RV) can occur as the RV responds to and compensates for the elevated pulmonary arterial pressure. In the present study, we observed increased pulmonary congestion after 28 days of TAC in the CMKO mice compared to MLK3 intact littermates as well as corresponding RV hypertrophy (Fig. 3.7A). We therefore interpret these findings to support the further role CM-specific MLK3 in maintaining normal cardiac function in the setting of cardiovascular stress.

4.5 CM-specific MLK3 deletion induces pathologic gene expression

In vitro analysis using MLK3 fl/fl left ventricle tissue isolated 28 days after TAC showed pathological gene expression in CMKO mice compared to MLK3 intact littermates. Fetal genes including ANP and BNP are typically expressed at low levels in the adult heart but can become re-expressed in response to pathological conditions including cardiac hypertrophy. Increased filling pressures, which lead to elevated myocyte stretch, also promote ANP and BNP release and re-expression. So, although ANP and BNP are believed to be compensatory in HF, they also serve as sensitive markers of pathological hypertrophy\(^{36}\). We observed a large increase in ANP expression
in the CMKO mice after TAC compared to MLK3 intact littermates, however, BNP expression increased to a similar extent in both MLK3 fl/fl TAC groups regardless of genotype (Fig. 3.8A). CTGF is a signaling protein involved in several processes including cell adhesion and migration, angiogenesis, myofibroblast activation, and extracellular matrix deposition. Because of these functions, CTGF is a sensitive molecular marker of remodeling and fibrosis. CTGF was increased in both TAC groups, but to a greater extent in the CMKO mice compared to MLK3 intact littermates (Fig. 3.8B).

In the 2019 MLK3 global deletion study, consistent with the present study, ANP and CTGF both increased in the MLK3 KO mice compared to wildtype littermates after TAC. However, BNP also increased in MLK3 KO over wildtype littermates after TAC which we did not observe in the CMKO TAC mice. This could be due to differences in the function of MLK3 globally versus in the CM, differences in the severity of TAC, or simply due to differences in sample sizes. Another notable difference is the scale of ANP expression in the MLK3 disruption group. We observed approximately a 20-fold increase in ANP in the MLK3 CMKO mice after TAC relative to the MLK3 intact sham group. ANP expression in the MLK3 KO mice showed an approximate 5-fold increase over the wildtype sham group. This is likely due to the more severe TAC model used in the present study. Taken together, therefore, our findings support a requirement of CM MLK3 in normally attenuating pathological gene expression in the LV after pressure overload.
4.6 CM-specific MLK3 deletion leads to increased myocardial interstitial fibrosis after TAC

After 28 days of TAC, we observed increased myocardial interstitial fibrosis in CMKO mice compared to MLK3 intact littermates and increased perivascular fibrosis both CMKO and MLK3 intact mice (Fig. 3.9A). Interstitial fibrosis is characterized by an increase in extracellular matrix and collagen deposits throughout the myocardium. Perivascular fibrosis is characterized by the deposition of connective tissue surrounding vessels. Both perivascular and interstitial fibrosis can occur in response to hypertrophy. Additionally, interstitial fibrosis has been identified in dilated cardiomyopathy. Perivascular fibrosis may progress to interstitial fibrosis and can occur in response to pathologic stress such as volume or pressure overload. There is evidence that inflammatory signaling around the perivascular spaces could be involved in this process.

Previous observations in the MLK3 global KO mouse identified no effect of MLK3 deletion on interstitial or perivascular fibrosis after TAC, which were attributed to a minimal effect of MLK3 on LV fibrosis in pressure overload. By contrast, we observed increased interstitial fibrosis only in the MLK3 CMKO mice after TAC. MLK3 intact littermates after TAC and both sham groups exhibited low levels of interstitial fibrosis. As mentioned above, the MLK3 disruption model and severity of TAC differed between the two studies. Another potential explanation for these differing results could be that MLK3 plays opposing or cell-specific roles on myocardial. Future studies will address these questions.

4.7 CM-specific MLK3 activity after pressure overload is independent of blood pressure effects
Previous MLK3 genetic loss of function studies\textsuperscript{17, 25} used global disruption approaches. Mice with disrupted MLK3 exhibited increased LV dysfunction and hypertrophy but also had basal elevated blood pressure. This suggests a role of MLK3 in both cardioprotective preservation of LV structure and function, but also a role in lowering blood pressure. Further, these observations suggest the possibility that the observed hypertension predisposes the heart to damage, contributing to the LV abnormalities. The signaling molecule PKG1 is an upstream regulator of MLK3 and also has a blood pressure lowering function through vasodilation\textsuperscript{12}. However, PKG1 attenuation of LV dysfunction requires MLK3 whereas PKG1 reduction of blood pressure does not. For example, after TAC, sildenafil improves pulmonary congestion and LV function in wild type mice but not in MLK3 KO mice. By contrast, the PKG1 activating drugs sildenafil or BAY41-2272 reduce blood pressure equally in both MLK3 KO and wildtype mice. Finally, inhibition of RhoA normalizes blood pressure in MLK3 KO mice and pharmacological inhibition of MLK3 in wildtype mice did not increase blood pressure. These collective observations support that MLK3 normally represses increases in blood pressure through kinase independent inhibition of RhoA\textsuperscript{24}. Despite these findings, it remains a possibility that the pathological LV functional phenotype in the whole-body MLK3 mutants arises to some degree from the pre-existing hypertension. In the present study, we found that CM-specific MLK3 deletion resulted in LV dysfunction and eccentric remodeling after TAC. Additionally, direct hemodynamic analysis shows no significant difference in systolic blood pressure or developed pressure in CMKO and MLK3 intact sham groups (Fig. 3.5A). Taken together, these results support the hypothesis and we conclude that CM-specific MLK3 normally opposes LV dysfunction and eccentric remodeling after pressure overload through a blood pressure independent mechanism.
4.8 Clinical relevance

The findings from our study support that in the setting of pressure overload, MLK3 prevents LV eccentric hypertrophy, preserves LV contractile function, and opposes myocardial interstitial fibrosis, through a role in the CM. Our previous work in global MLK3 KO mice demonstrated that MLK3 is required for the therapeutic effects of sildenafil in TAC. Sildenafil normally opposes HF through activation of the cGMP dependent protein kinase 1 (PKG1). MLK3 is a direct substrate effector of PKG1α.24 PKG1 activating drugs show improved outcomes in HFrEF40,41 but are frequently limited by the side effect of hypotension.40,41,42 Because our current study now identifies that MLK3, in the CM, opposes LV dysfunction through blood pressure-independent effects, this raises the possibility that pharmacological augmentation of MLK3 could represent a strategy to promote PKG1 LV therapeutic effects in HFrEF without adverse hypotensive effects.

4.9 Limitations

The current study has several limitations. First, only male mice were investigated. Initially, we planned to examine the effects of CM-specific MLK3 deletion in both male and female mice but were limited by time constraints. Further study is therefore necessary to extend our conclusions to female mice. Additionally, we note that some of the findings in the no-flox control mice are limited by relatively small sample size. This occurred due both to time constraints and to the difficulty of obtaining complete hemodynamic analysis in some mice with more severe phenotypes. Furthermore, we note that this study investigated two separate strains of mice. Specifically, we compared littermates within MLK3-floxed and MLK3 no-flox strains, but we were not able to compare littermates between the two strains. Thus, our exploratory direct comparison of
MLK3 fl/fl and non-floxed mice is potentially limited by strain differences. For these reasons, it remains a formal possibility that the non-MLK3-related toxic effects of Cre drove the phenotypic changes observed even in the MLK3 fl/fl x Cre mice. However, observed no significant baseline strain differences in the wildtype Cre- non-floxed and MLK3 intact mice, making this possibility much less likely. Finally, in the present study we describe the effects of CM-specific MLK3 deletion in mice, however the effect in other species is unknown. Further investigation is required to translate these findings to other species including humans.
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