Prenatal versus postnatal regulation of blood pressure and cardiac phenotype by MLK3

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Abstract

Cardiovascular disease remains a leading cause of morbidity and mortality worldwide. Of the many cardiovascular disorders, hypertension represents a highly prevalent condition which contributes to myocardial infarction, stroke, heart failure, and many other lethal conditions. Recent studies have identified Mixed Lineage Kinase 3 (MLK3) as a novel substrate of PKG1α, a key regulator in cardiovascular function. MLK3 has been implicated specifically in maintaining vascular homeostasis, opposing cardiac dysfunction under conditions of left ventricular pressure overload, and playing a crucial role in blood pressure regulation. Germline global MLK3 deletion in mice resulted in significant hypertension and reduced resistance vessel distensibility, highlighting its importance in blood pressure control and in vascular tone modulation. Prior research found that global MLK3 deficiency leads to abnormal smooth muscle cell proliferation and neointima formation following carotid injury. Additionally, MLK3 global deletion mice develop baseline left ventricular (LV) hypertrophy, a common precursor to heart failure. In humans, MLK3 polymorphisms have been associated with age-related increases in blood pressure, further supporting the relevance of MLK3 to blood pressure regulation. These findings suggest that MLK3 exerts complex regulatory effects on cardiovascular physiology, influencing

both myocardial structure and function and vascular dynamics.

While MLK3 plays a crucial role in modulating blood pressure and cardiac structure as described above, whether these effects occur due to a developmental role of MLK3 or instead through postnatal effects on physiology remains unclear. The effects of postnatal MLK3 deletion on blood pressure and cardiovascular physiology remain unstudied. We therefore generated a whole-body inducible MLK3 gene knockout (iKO) mouse model using a whole body-inducible Cre-loxp system, and determined the effects of postnatal inducible MLK3 whole-body knockdown on basal blood pressure and cardiac structure and function. Western blot analysis confirmed significant MLK3 knockdown in lung and spleen tissue of MLK3 iKO compared with MLK3 intact mice, supporting effective gene deletion. Compared with MLK3 intact littermate controls, MLK3 iKO mice displayed increased in blood pressure, as assessed through implanted telemetry. Notably, the hypertensive phenotype was observed during both light and dark phases, suggesting that MLK3 is essential for maintaining normal blood pressure homeostasis during rest and active periods By contrast, left ventricular structure, mass, or diastolic function did not differ between MLK3 intact and iKO littermates, indicating that the increased blood pressure is independent of major structural cardiac changes. Unexpectedly, MLK3 iKO spleen mass normalized to tibia length was reduced in iKO mice

compared with MLK3 intact controls, while other organs remained unaffected. Finally, our results suggest a potential sex-dependent effect of MLK3 on blood pressure regulation, as male MLK3 knockout mice exhibited a greater hypertensive response than females in a MLK3 germline knockout model, but not in the MLK3 inducible knockout mice. These findings highlight the critical role of MLK3 in postnatal blood pressure regulation and suggest that its function may be mediated through a yet-to-be-determined cell type.

We conclude that MLK3 plays a critical role in regulating blood pressure, and that it likely regulates cardiac function through distinct mechanisms independent of its blood pressure effects. These findings support further investigation of MLK3 postnatal blood pressure regulation in order to find novel candidate therapeutic targets for hypertension.

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List of Abbreviations

ACE	Angiotensin converting enzyme
ARBs	Angiotensin II receptor blockers
BW	Body Weight
CM	Cardiomyocyte
DBP	Diastolic blood pressure
EF	Ejection fraction
FS	Fractional shortening
gKO	germline Knock-out
HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
HR	Heart rate
iKO	inducible Knock-out
IVS;d	Left Ventricular Internal Diameter in Diastole
JNK	c-Jun N-terminal kinase
KO	Knock-out
LA	Left Atrium
LV	Left ventricular
LVH	Left ventricular hypertrophy
LVID;s	Left Ventricular Interin Diastole

LVPW;d	Left Ventricular Posterior Wall in Diastole
LZ	Leucine zipper
MAP	Mean blood pressure
MAPK	Mitogen-activated protein kinase
MKK	Mitogen activated protein kinase kinase
MLK3	Mixed lineage kinase 3
PKG1	cGMP-dependent protein kinase 1
RA	Right atrium
RV	Right ventricular
SBP	Systolic blood pressure
TBST	Tris-buffered saline with 0.1% Tween 20
TL	Tibia length

Chapter 1: Introduction

1.1 Hypertension

Hypertension is one of the most significant preventable risk factors for cardiovascular disease worldwide （Mills et al., 2020）, with its prevalence continuing to rise, particularly in low- and middle-income countries (LMICs), driven by aging populations, urbanization, and lifestyle changes. The development of hypertension involves a combination of genetic and environmental factors. The 2017 ACC/AHA guidelines highlight the significant impact of dietary habits, with high sodium and low potassium intake being key modifiable risk factors (Whelton et al., 2018). Additionally, obesity, sedentary lifestyles, high-fat and high-calorie diets, excessive alcohol consumption, and smoking are closely associated with hypertension. Psychological stress also plays a role by activating the sympathetic nervous system and increasing cortisol levels, leading to vasoconstriction and elevated blood pressure. In some cases, hypertension results from underlying conditions such as chronic kidney disease, primary aldosteronism, or Cushing's syndrome, requiring targeted treatment.

Uncontrolled hypertension significantly increases the risk of cardiovascular diseases and damages multiple organ systems. A study by

Lewington et al. (2002) involving over one million individuals demonstrated a dose-response relationship between elevated blood pressure and cardiovascular mortality, with every 20 mmHg increase in systolic pressure or 10 mmHg increase in diastolic pressure doubling the risk of cardiovascular disease. Prolonged hypertension can lead to left ventricular hypertrophy and cardiac remodeling, ultimately resulting in heart failure. It is also a major risk factor for chronic kidney disease, as persistent high blood pressure causes glomerular hyperfiltration and vascular damage, leading to kidney failure. Hypertension significantly increases the likelihood of myocardial infarction and stroke, with research identifying it as the leading risk factor for stroke (GBD 2019 Risk Factors Collaborators, 2020). Furthermore, prolonged hypertension exerts mechanical stress on the aortic wall, promoting aneurysm formation and increasing the risk of aortic dissection, which carries a high fatality rate.

The management of hypertension primarily includes lifestyle modifications and pharmacological treatment to lower blood pressure and reduce the risk of complications (Williams et al., 2018). Lifestyle modifications are recommended for all hypertensive patients and include reducing sodium intake, increasing potassium intake, following a healthy diet (such as the DASH diet), maintaining a healthy weight, engaging in regular physical activity, limiting alcohol consumption, and quitting

smoking. If lifestyle changes are insufficient to control blood pressure, antihypertensive medications such as ACE inhibitors, ARBs, calcium channel blockers, and diuretics may be prescribed, either individually or in combination, to achieve optimal blood pressure control.

Despite established hypertension management strategies, global blood pressure control rates remain low, particularly in LMICs. Future research should focus on optimizing prevention and treatment strategies, improving patient adherence, and assessing the long-term impact of hypertension on global healthcare systems and economies. Early screening and intervention for high-risk populations are also essential to reduce the burden of hypertension-related diseases and lower the incidence and mortality of cardiovascular conditions worldwide.

1.2 Heart failure

Heart Failure (HF) is a common consequence of hypertension and represents the leading cause of death and hospitalization among adults in the United States, with a lifetime risk estimated at 24% (Bozkurt et al, 2023). Risk factors for HF include hypertension, as well as left ventricular (LV) hypertrophy, obesity, diabetes mellitus, and renal insufficiency, all of which contribute to its progression. HF is broadly

classified based on left ventricular ejection fraction (EF) into two major categories: heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF). HFrEF, defined by an EF ≤ 40%, is often caused by cardiomyocyte loss due to ischemia, genetic mutations, myocarditis, or valvular disease, leading to systolic dysfunction and impaired ventricular contraction. In contrast, HFpEF is characterized by an EF ≥ 50% and is associated with chronic comorbidities such as hypertension, Type 2 diabetes mellitus(T2DM), obesity, and inflammation, resulting in ventricular stiffness, fibrosis, and impaired relaxation. HFpEF has become increasingly prevalent, now accounting for over 50% of all HF cases, yet remains without effective treatment options. While significant advances have improved outcomes for HFrEF through neuro-humoral axis-targeting therapies such as β-blockers, angiotensin-converting enzyme (ACE) inhibitors, and angiotensin II receptor blockers (ARBs), similar approaches have failed for HFpEF, underscoring the need for further research into the distinct molecular mechanisms driving these two conditions.

1.3 Identification of mixed lineage kinase 3 as a novel effector of protein kinase G 1 alpha.

Over the past several decades, the signaling molecule

cGMP-dependent protein kinase 1α (PKG1a) has been implicated as a both a critical regulator of blood pressure and as opposing cardiac dysfunction in heart failure. Its activation through cGMP-elevating agents, such as sildenafil, guanylate cyclase stimulators, and neprilysin inhibitors (sacubitril/valsartan) has shown promise in heart failure (HF) treatment by mitigating LV remodeling, preserving cardiac function, and improving clinical outcomes. However, the therapeutic potential of PKG1α activation is often constrained by its vasodilatory effects, which can lead to hypotension. Interest has grown, therefore, in understanding the specific downstream PKG1a kinase substrates and effectors which mediate PKG1 effects on vasodilation and on cardiac biology. For example, PKG1a substrates such as RhoA, regulator of G protein signaling 2, transient receptor transient receptor potential cation channel subfamily C 6 (TRPC6) and myosin phosphatase have been identified which in the vascular smooth muscle cell (VSMC) promote vasorelaxation but in the cardiac myocyte affect contractile function.

Other PKG1a substrates have been identified which function only in the cardiac myocyte or in striated muscle cells, but not in the VSMC. For example, PKG1a directly binds and phosphorylates sarcomere-associated proteins cardiac troponin I and cardiac myosin binding protein-C, both of which alter CM systolic and diastolic function in response to direct phosphorylation by PKG1a. These and other studies support that PKG1a

exerts direct effects on cardiac structure and function through mechanisms independent of its effect on blood pressure.

In addition to identifying some of the molecules described above as PKG1a effectors, the Blanton laboratory has further explored the molecular mechanisms through which PKG1a separately regulates cardiac and blood pressure effects in vivo. A key finding from recent studies is the identification of MLK3 (mixed lineage kinase 3) as a novel PKG1α effector that mediates its cardioprotective effects while independently regulating vascular tone.

MLK3 directly interacts with PKG1α via its leucine zipper (LZ) domain and is phosphorylated by PKG1a on Thr277/Ser281, a modification essential for its kinase activity. MLK3 is crucial in mediating PKG1a effects on preserving LV function under conditions of pressure overload, as evidenced by studies in global MLK3-knockout mice, where the absence of MLK3 abolishes the cardioprotective effects of PKG1α activation. Additionally, MLK3 deletion leads to increased vascular stiffness and chronic hypertension, yet does not interfere with the acute BP-lowering effects of PKG1α activation, indicating that MLK3 controls BP via a PKG-independent mechanism. These findings suggest that MLK3 could serve as a therapeutic target to selectively enhance the beneficial effects of PKG1α on the heart while minimizing its hypotensive drawbacks.

1.3 MLK3 in regulating blood pressure

The Blanton laboratory interest in MLK3 arose from its role as a PKG1a effector. However, one active area of investigation is understanding the unexpected finding that germline MLK3 global deletion mice display significant hypertension. Our lab demonstrated that whole-body MLK3 knockout mice develop systemic hypertension as early as two months of age, which is associated with reduced vascular distensibility and reduced size of VSMCs. VSMCs isolated from MLK3 germline deletion mice (denoted here as gKO) also display evidence of RhoA hyperactivity. Similarly, mice with whole body knockin mutation disrupting the Cdc42-interaction domain of MLK3 also display hypertension until at least 6 months of age. Recent studies have highlighted the broader impact of MLK3 deletion on cardiovascular function, revealing its critical role in maintaining vascular integrity. Additionally, MLK3-deficient mice exhibit abnormal smooth muscle cell proliferation following vascular injury, leading to neointimal thickening and an increased risk of vascular occlusion. Taken together these findings reveal that MLK3 regulates basal blood pressure and also that deletion of MLK3 produces abnormalities of VSMC structure, resistance vessel phenotype, and vascular response to injury.

Blood pressure is regulated through a complex interplay between various organs and tissues, each contributing to maintaining vascular homeostasis. The kidneys play a crucial role in fluid and electrolyte balance through the renin-angiotensin-aldosterone system (RAAS), adjusting blood volume and systemic resistance. Blood vessels, specifically vascular smooth muscle cells (VSMCs), regulate vessel tone through vasoconstriction and vasodilation, which influence peripheral resistance. The brain and nervous system control autonomic regulation via sympathetic and parasympathetic pathways, modulating heart rate and vascular tone. Additionally, the immune system has emerged as a significant regulator, with inflammatory cytokines affecting endothelial function and vascular remodeling, contributing to hypertension development.Given the critical role of vascular smooth muscle cells in blood pressure regulation, MLK3 has been identified as a key kinase involved in maintaining vascular integrity and modulating blood pressure. Its function in smooth muscle contraction and vascular resistance suggests a potential link to hypertensive mechanisms. Previous studies have demonstrated that MLK3 is involved in VSMC function by modulating myosin light chain (MLC) phosphorylation, which is essential for smooth muscle contraction and vascular resistance. Moreover, the deletion of MLK3 in animal models has been associated with vascular abnormalities, including increased smooth muscle proliferation and

neointima formation, which contribute to elevated blood pressure. Additionally, MLK3 has been linked to PKG1 (Protein Kinase G1) signaling, a key modulator of VSMC relaxation. Given these findings, our study aimed to explore how MLK3 influences blood pressure regulation through its effects on vascular smooth muscle cells. Interestingly, despite the critical role of MLK3 in VSMC function, our findings indicate that MLK3 knockout in smooth muscle cells alone did not significantly impact systemic blood pressure. This suggests that MLK3 may have broader effects on blood pressure regulation beyond VSMCs. Future investigations should explore MLK3's role in other key regulatory systems, such as renal function, endothelial cell signaling, and neurovascular interactions, to fully elucidate its contribution to blood pressure homeostasis.

The absence of an inducible blood pressure effect in smooth muscle cell (SMC)-specific MLK3 knockout mice suggests that MLK3's influence on hypertension may extend beyond SMCs. This observation raises the possibility that MLK3 deletion leads to hypertension due to its role during prenatal or embryological development. Supporting this notion, a study by Ramo et al. (2016) demonstrated that MLK3 is essential for proper vascular morphogenesis during development. MLK3 deficiency resulted in defective collateral artery formation, leading to impaired blood perfusion and increased tissue injury following arterial

occlusion. Furthermore, research has identified polymorphisms in the MAP3K11 gene, which encodes MLK3, associated with hypertension in certain populations, suggesting a genetic predisposition linked to MLK3 variants. MLK3's role extends to cardiac function, particularly under conditions of pressure overload. In a study by Calamaras et al. (2019), MLK3 knockout (KO) mice subjected to transverse aortic constriction (TAC) exhibited increased left ventricular (LV) hypertrophy and dysfunction compared to wild-type controls. This suggests that MLK3 serves as a protective factor against adverse cardiac remodeling. However, it is important to note that the observed LV hypertrophy in MLK3 KO mice could be secondary to chronic hypertension present before TAC surgery. This pre-existing hypertension may exacerbate the cardiac response to pressure overload, indicating that MLK3's absence affects both blood pressure regulation and cardiac structural integrity. To delineate MLK3's specific contributions, further research is needed to assess whether postnatal MLK3 directly regulates both blood pressure and basal LV structure and function, independent of developmental influences.

Interestingly, while MLK3 deletion in smooth muscle cells alone does not result in hypertension (unpublished results, Blanton Laboratory). Whole-body MLK3 knockout leads to elevated blood pressure, suggesting that MLK3 may regulate blood pressure through other cell

types, such as endothelial cells or even the central nervous system. Given that MLK3 polymorphisms have been associated with age-related hypertension in humans, another possibility is that MLK3 only regulates blood pressure because of some effect on development. To investigate this, we utilized the Cre-loxP system to generate an MLK3 gene knockout mouse model, allowing us to explore whether its effects on blood pressure are developmentally programmed or mediated through specific cell types.

1.4 MLK3 in cardiac hypertrophy, remodeling, and heart failure

Left ventricular hypertrophy (LVH) is a pathological condition characterized by an increase in left ventricular mass due to cardiomyocyte enlargement. It often occurs as an adaptive response to increased workload, such as pressure or volume overload. While initially compensatory, prolonged LVH can lead to adverse cardiac remodeling and heart failure.

Mixed lineage kinase 3 (MLK3) is a serine/threonine kinase belonging to the mitogen-activated protein kinase (MAPK) family and plays a crucial role in cellular signaling pathways, particularly in cardiovascular function. MLK3 interacts with small GTPases like cdc42 and activates mitogen-activated protein kinase kinase-4 (MKK4) and MKK7, leading

to the stimulation of c-Jun N-terminal kinase (JNK) signaling. This JNK activation is implicated in various cellular processes, including growth, differentiation, and apoptosis, and has been shown to counteract concentric left ventricular hypertrophy (LVH). The protective role of MLK3 in the myocardium has been demonstrated in experimental models, where its deletion leads to aggravated cardiac remodeling, increased LV dimensions, and worsened systolic function following pressure overload. MLK3 is also necessary for the cardioprotective effects of PKG1α activation, as seen in studies where sildenafil, a PKG1α activator, failed to improve heart failure outcomes in MLK3 knockout (KO) mice.

Beyond its role in JNK signaling, MLK3 also regulates vascular function by modulating the activity of RhoA, a small GTPase involved in smooth muscle contraction and vascular tone regulation. MLK3 inhibits RhoA through an allosteric, kinase-independent mechanism, preventing excessive vascular contraction and maintaining proper blood pressure homeostasis. Loss of MLK3 leads to increased RhoA activation, which contributes to vascular stiffness and hypertension. Despite this, acute blood pressure-lowering effects of PKG1α activation remain unaffected in MLK3-deficient mice, suggesting that MLK3 independently modulates vascular function without interfering with PKG1α-mediated vasodilation. Pharmacological inhibition of MLK3 in wild-type mice does not alter systemic blood pressure, further confirming that its effects on RhoA are

specific to long-term vascular remodeling rather than acute hemodynamic regulation.

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 whole body inducible knockout (iKO) mice and MLK3 intact littermates were obtained by breeding mice harboring LoxP sites flanking the MAP3K11 gene encoding MLK3 (MLK3fl/fl) with Ubiquitin C-Cre (UBC-Cre)transgenic mice in the animal facility at Tufts Medical Center. MLK3fl/fl mice were generated in partnership with Ingenious Targeting Laboratory. UBC-Cre mice were obtained from The Jackson Laboratory. Non-floxed controls were obtained by breeding MLK3 fl/+ mice with MLK3 fl/+; UBC-Cre+ mice. The UBC-Cre transgene was introduced into the genome through pronuclear microinjection or targeted knock-in strategies, and the mice were maintained on a C57BL/6 background.

2.3 Tamoxifen injection

Tamoxifen was used for MLK3 gene knockout. 0.1grams of tamoxifen was dissolved in 1ml of 100% Ethanol (run under warm water to dissolve). Subsequently, 9 ml of sunflower seed oil was added, and the mixture was transferred to a Falcon tube, covered with tinfoil, and placed on a shaker in a cold room for 2 hours to ensure thorough mixing. For administration, intraperitoneal (IP) injections of 100 µl were given once daily for 5 consecutive days to mice aged 6–8 weeks.

2.4 Surgery and Blood pressure measurement

To measure blood pressure in mice, surgically implanted telemetry transmitters were used for continuous and precise monitoring. Mice were anesthetized with isoflurane (2.5% for induction, 1.5% for maintenance) and placed on a heating pad to maintain body temperature. Following aseptic preparation of the surgical site, a small incision was made to expose the abdominal aorta. The catheter of the telemetry transmitter was carefully inserted and secured in the aorta using medical-grade adhesive, while the transmitter body was positioned subcutaneously within the abdominal cavity. The incision was then closed with sutures, and mice

were monitored closely during recovery, with postoperative analgesia administered to minimize discomfort.

After a 1-week recovery period to ensure stable physiological conditions, continuous blood pressure monitoring commenced. The telemetry system recorded systolic, diastolic, and mean arterial pressure, as well as heart rate and activity levels. Data collection was conducted over a 48-hour period, encompassing both light and dark cycles to assess circadian fluctuations in blood pressure. This approach enables high-resolution, real-time monitoring, providing a comprehensive assessment of blood pressure dynamics under experimental conditions.

2.5 Echocardiography

Cardiac function in mice was assessed using echocardiography, as previously described (3,4). For this study, echocardiograms were performed one day before organ harvest. Mice were anesthetized with 2.5% gaseous isoflurane in medical oxygen at 1 L/min and maintained under 1%–2% isoflurane while positioned supine on a 39°C heating pad. Chest fur was removed using a depilatory cream (Nair), and any excess was wiped off with a damp cotton pad. Ultrasonic gel was then applied to the echocardiography transducer (MS550D; Vevo2100, FUJIFILM Visual

Sonics), and scanning was performed in both the long- and short-axis views using M-mode and B-mode imaging.

M-mode images were acquired from the mid-papillary short-axis view. Mitral inflow velocity was measured using pulse-wave Doppler, while septal annular e'velocity was obtained via tissue Doppler. All Doppler measurements were performed from the apical view. Echocardiographic data acquisition and analysis were conducted by a single blinded investigator.

2.6 Tissue isolation

Following echocardiography, the heart, liver, lungs, spleen, kidneys, and aorta were collected, and tibia length was measured. Each organ was then weighed, flash-frozen in liquid nitrogen, and stored at -80°C.

2.7 Immunoblotting

The protein from the heart, liver, lungs, spleen, kidneys, and aorta of control and Mlk3 knockout mice was extracted using T-PER tissue lysate supplemented with 1 mol/L EDTA, protease, and phosphatase inhibitors. Protein concentration was determined using the Bicinchoninic Acid (BCA) Assay Kit (ThermoFisher Scientific).

For lung, spleen, and aorta tissues, homogenization was performed on dry ice by pulverization using a stainless steel mortar and pestle. The resulting tissue powder was lysed with 200 µL tissue lysis buffer per 10 mg of tissue, consisting of 20 mM HEPES, 50 mM β-Glycerol Phosphate, 2 mM/L EGTA, 1 mM/L DTT, 10 mM/L NaF, 1 mM/L NaVO4, 1% Triton-X 100, and 10% Glycerol. The mixture was vortexed for 20 seconds per sample and kept on ice for 1 hour, with agitation every 10–20 minutes. After centrifugation, the supernatant was collected for further analysis.

Protein samples were diluted 1:1 with 2x Laemmli Sample Buffer containing SDS (Sigma S-3401) and vortexed before being boiled at 100°C to denature the proteins. Protein samples and a protein ladder marker (Bio-Rad Precision Plus Dual Color Standards) were loaded into 4-15% polyacrylamide gels and separated using SDS-PAGE running buffer (ChemCruz; 10X running buffer) for approximately 2 hours. Proteins were then electrotransferred onto nitrocellulose membranes (Bio-Rad, 1620094) in transfer buffer (5x Turbo Transfer Buffer 20%, distilled water 60%, methanol 20%). Transfer efficiency was confirmed using Ponceau S staining, followed by blocking with 5% Bovine Serum Albumin (BSA).

The membranes were incubated overnight at 4°C with the primary antibodies, including anti-MLK3 (1:300 in 5% Bovine Serum Albumin (BSA)/TBST; Abcam, Ab51068) and Vinculin (1:1000 in 5% BSA/TBST; Cell Signaling Technology). After primary antibody incubation, membranes were treated with secondary antibody (1:1000 in 5% BSA) for 1 hour at room temperature. Following brief washes in TBST, protein bands were visualized using enhanced chemiluminescence (ECL) substrate (ThermoFisher Scientific, 34095) and imaged using the ProteinSimple FluorChem E system to confirm protein expression levels.

2.8 Statistical analysis

All data are presented as means ± SE, and the statistics were performed with Student's two-tailed unpaired t test. For comparing more than two groups, we used two-way ANOVA with Sidak's multiple-comparisons test. Values of $P < 0.05$ were considered statistically significant.

2.9 Contributions

All surgeries were performed by Gregory Martin. The Study planning, animal husbandry, tamoxifen injection, echocardiography, blood pressure measurement, tissue isolation, immunoblot, and all data analysis were

performed by Yi Pan.

Chapter 3 Results

3.1 Confirmation of MLK3 knockdown in inducible MLK3 whole-body KO mice

To generate inducible MLK3 whole-body knockout (KO) mice, tamoxifen was administered intraperitoneally (IP) at a concentration of 10 mg/mL in 100 μL volumes for five consecutive days starting at 6–8 weeks of age. Following tamoxifen injection, mice were maintained for an additional 4 weeks to allow for complete recombination and MLK3 deletion. To confirm successful MLK3 deletion, Western blot analysis was performed

on various tissue lysates including lung, spleen, and aorta. Quantification of MLK3 expression relative to vinculin demonstrated a significant reduction in MLK3 levels in lung and spleen tissues of MLK3 KO mice compared to wild-type controls, confirming effective gene deletion and protein knockdown. (Figure 3.1 A, B). However, MLK3 knockdown in aortic tissue lysates (Figure 3.1 C) did not result in a significant change, indicating a tissue-specific variation in MLK3 expression.

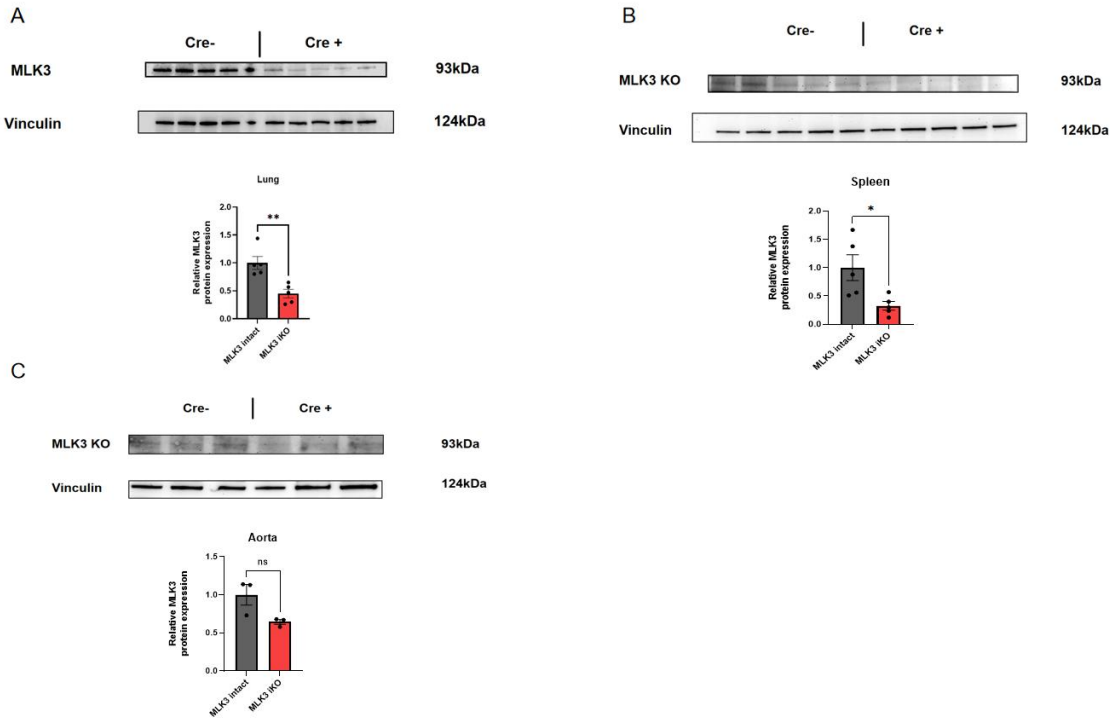


Figure 3.1 Confirmation of MLK3 knockdown in inducible whole-body MLK3 KO mice. Immunoblot of MLK3 and vinculin loading control in protein lysates of: (A) lung, (B) spleen (C) aorta, from MLK3 intact (MLK3fl/fl;Cre−, n=5 for lung and spleen; n=3 for aorta) and MLK3 iKO (MLK3fl/fl;Cre+, n=5 for lung and spleen; n=3 for aorta) mice. Quantification of MLK3 protein expression relative to Vinculin by densitometry is shown in the bar graph. *, $p<0.05$; **, $p<0.001$. Data are presented as mean ± SEM, and analyzed by two-tailed unpaired t-test.

3.2 MLK3 inducible whole-body knockdown leads to hypertension in 12-14 week-old mice.

To investigate the effect of MLK3 gene deletion on blood pressure, we performed implantable telemetry and measured 48 hour blood pressure, heart rate, and activity in adult (12–14 weeks old) MLK3 inducible knockout (iKO) mice. We used MLK3 germline deletion (gKO) mice as positive controls, as we previously demonstrated hypertension in these mice. The basal systolic blood pressure (SBP) and mean arterial pressure (MAP) of MLK3 iKO mice were significantly higher than those of the intact mice, while diastolic blood pressure (DBP) showed no significant difference (Figure 3.2A). These results indicate that whole-body postnatal MLK3 deletion leads to hypertension, consistent with the previously reported phenotype of the MLK3 gKO mice. To ensure that Cre expression alone did not affect blood pressure, we included a No-Flox Cre+ control group, which showed no significant differences compared to the intact mice. Based on this, we combined the No-Flox and intact groups into a single intact group for further analysis (Figure 3.2A).

Additionally, there were no significant differences in heart rate or activity levels between any of the experimental groups (Figure 3.2B), indicating that MLK3 deletion does not impact these parameters. To further assess the impact of MLK3 deletion on blood pressure regulation under different physiological conditions, we analyzed MAP during both the dark and light phases. The results were consistent with the 48-hour combined MAP measurements, showing that MLK3 gKO mice exhibited

significantly elevated blood pressure compared to the intact group in both phases (Figure 3.2C). However, no significant difference in MAP was observed between the iKO and gKO groups during the light phase. These findings suggest that the effect of inducible MLK3 deletion on blood pressure is comparable to that of germline MLK3 deletion during the light phase. By contrast, during the dark phase, MLK3 iKO MAP was not significantly increased versus intact, and was decreased versus gKO.

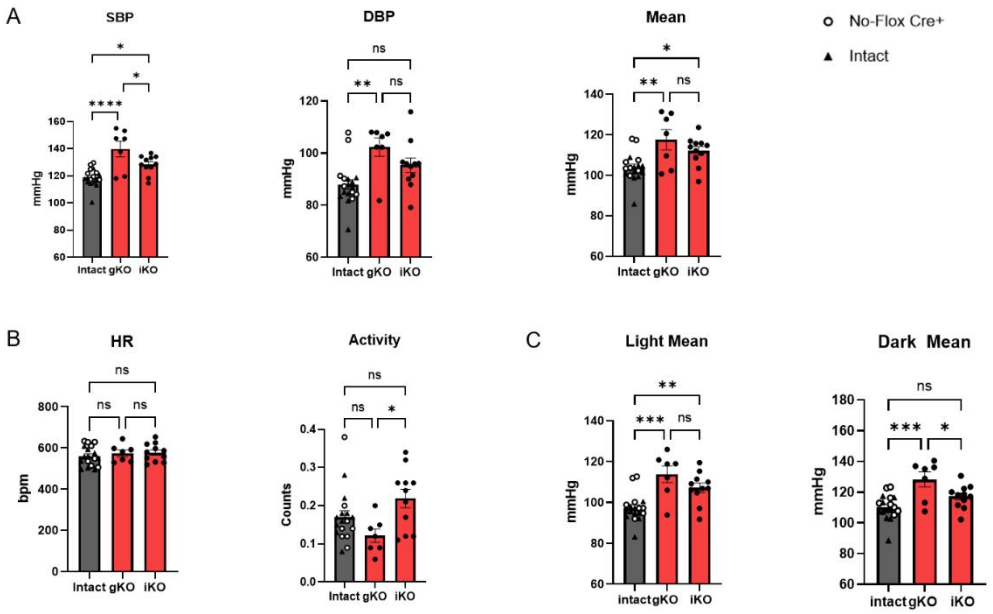


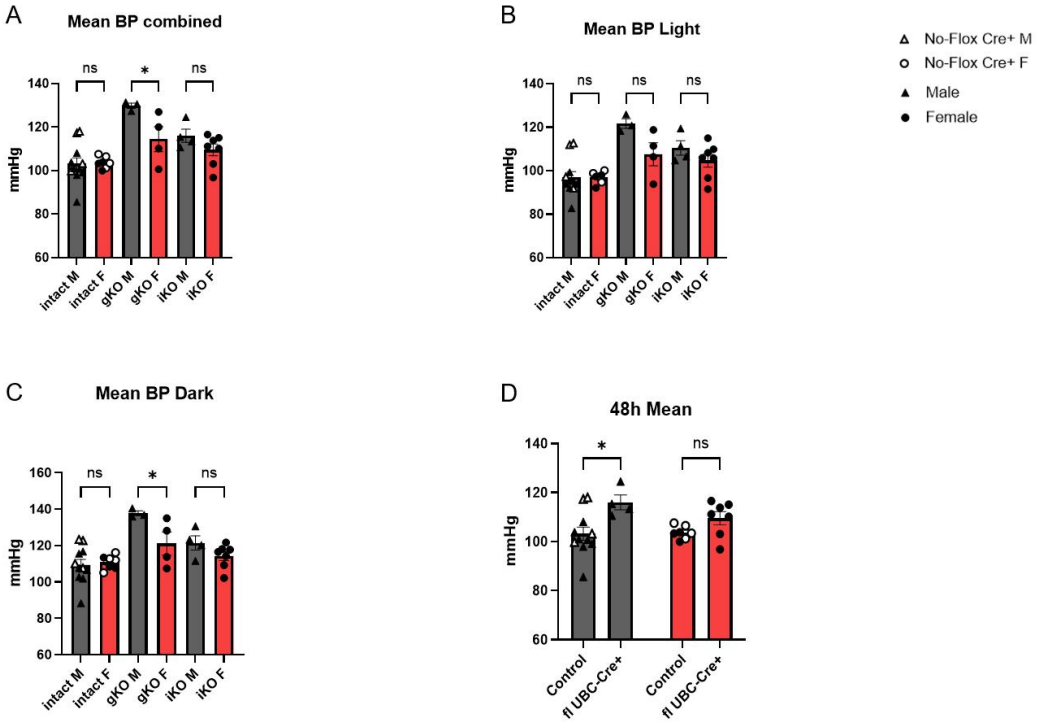
Figure 3.2 MLK3 inducible whole-body knockdown leads to hypertension in 12-14 weeks-old mice. (A)Systolic blood pressure (SBP), Diastolic blood pressure (DBP), Mean blood pressure, (B) heart rate (HR), and activity data collected by 48 hour implantable telemetry in conscious Intact controls (No-Flox Cre+, hollow circles; MLK3fl/fl; Cre-, triangles), germline KO (gKO), and inducible KO (iKO) mice. (C) The

same parameters separated by dark and light cycles. n= 19 intact (n=8 No-Flox Cre+; n=11 MLK3fl/fl), n=7 gKO, n=10 iKO. Data are presented as mean ± SEM. Statistical comparisons were by one-way ANOVA followed by Tukey's post-hoc test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = not significant

3.3 Sex-specific blood pressure effect in germline deletion but not inducible knockdown of MLK3.

To investigate potential sex effects of MLK3 in blood pressure regulation, we analyzed blood pressure data separately for male and female mice. In the gKO mice, male mice exhibited significantly higher blood pressure than females. By contrast, no significant sex differences were observed between other genotypes (Figure 3.3A, B, C). To further assess the effect of genotype on blood pressure independent of sex, we analyzed the combined the Cre+ No-Flox and the fl/fl Cre- groups as a single control "intact" group. Male MLK iKO mice exhibited significantly higher MAP compared to intact controls (P < 0.05), while no significant difference was observed between these genotypes in female mice (Figure 3.3D). Although a sex-based comparison within the KO group suggested a trend toward increased MAP in males, the overall analysis indicated that genotype had a significant effect on blood pressure,

whereas sex did not (Figure 3.3D). These findings suggest that MLK3 deletion contributes to elevated blood pressure, particularly in males, highlighting a potential sex-dependent variation in the hypertensive



phenotype.

Figure 3.3 Sex-specific blood pressure effect in germline deletion but not inducible knockdown of MLK3. (A, B, C) Mean blood pressure was measured in male (▲) and female (●) mice across different genotypes and conditions, including 48-hour averages, light-phase, and dark-phase measurements. Intact: Male n=11 (n=4 No-Flox Cre+, n=7 MLK3fl/fl), Female n=7 (n=4 No-Flox Cre+, n=3 MLK3fl/fl); gKO: Male: n=3, Female n=4; iKO: Male: n=4, Female: n=7. Data were analyzed using one-way ANOVA with Tukey's multiple comparisons test.

ns, $P \geq 0.05$; *, $P < 0.05$. (D) Mean blood pressure was measured in male and female mice in MLK3 intact and MLK3 iKO. Male iKO mice exhibited significantly higher MAP compared to intact ($P < 0.05$), while no significant difference was observed in females. Combined analysis showed a significant effect of genotype ($P = 0.0029$) but no significant effect of sex ($P = 0.3021$). Data were analyzed using two-way ANOVA with Šidák's multiple comparisons test. ns, $P \geq 0.05$; *, $P < 0.05$.

3.4 Long-term Influence of MLK3 Inducible Whole-Body Deletion on Blood Pressure in 6-Month-Old Mice.

To investigate the long-term effect of MLK3 deletion on blood pressure, we measured blood pressure and heart rate in 6-month-old MLK3 iKO and intact control mice over 48 hours, including both light and dark phases. Similar to the findings in younger adult mice, MLK3 iKO mice exhibited significantly higher mean arterial pressure (MAP) compared to the intact group ($P<0.05$), particularly during the light phase (Figure 3.4A, D), while no significant difference was observed during the dark phase (Figure 3.4C). Heart rate (HR) remained unchanged between groups (Figure 3.4B). These results indicate that MLK3 deletion leads to persistent hypertension in older mice, similar to younger adult mice, suggesting a sustained role of MLK3 in blood pressure regulation.

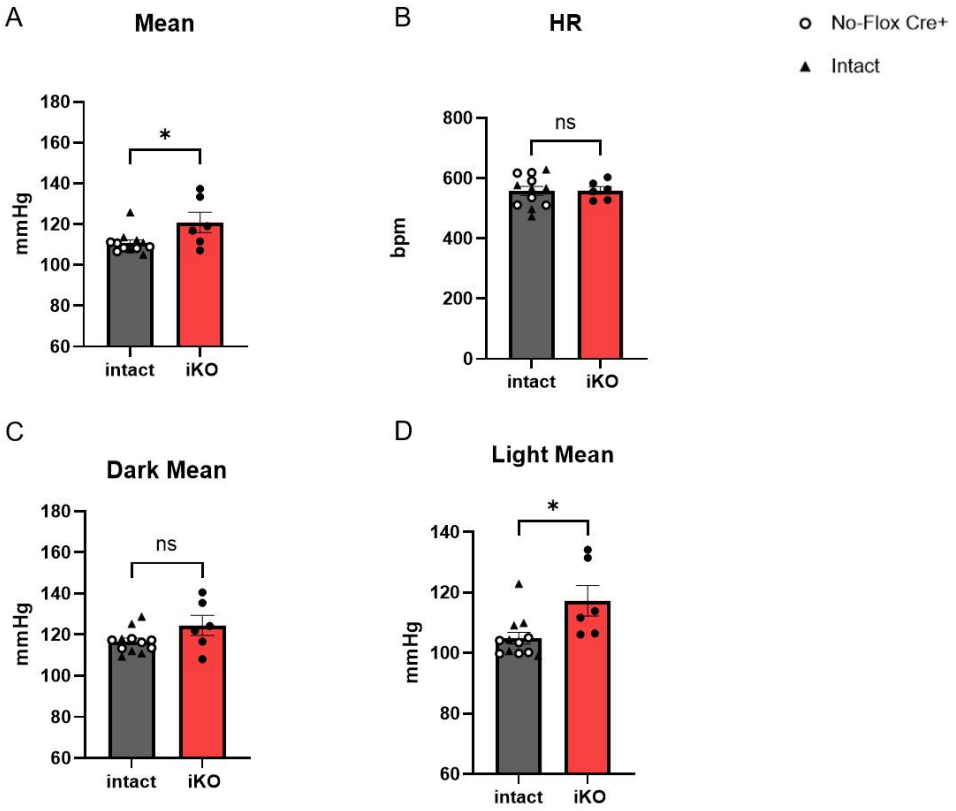


Figure 3.4 MLK3 inducible whole-body deletion leads to hypertension in 6 months-old mice.

(A) Mean blood pressure, (B) heart rate (HR), and activity data collected by 48 hour implantable telemetry in conscious Intact controls (No-Flox Cre+, hollow circles; MLK3fl/fl; Cre-, triangles) and MLK3 inducible KO (iKO) mice. (C, D) The same parameters separated by dark and light cycles. n=12 intact (n=6 No-Flox Cre+; n=6 MLK3fl/fl), n=6 iKO. Data are presented as mean ± SEM. Statistical comparisons were by one-way ANOVA followed by Tukey's post-hoc test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = not significant

3.5 Blood Pressure Over 48 Hours in Cre+ and Cre- Mice and comparison of Blood Pressure Between Light and Dark Cycles

To examine the changes in blood pressure over time between MLK3 intact and iKO mice, we continuously recorded arterial pressure over a 48-hour period. The time-course analysis (Figure 3.5A) revealed a distinct difference between the two groups, with MLK3 iKO mice exhibiting consistently higher blood pressure compared to intact mice throughout the measurement period. Both groups displayed characteristic circadian fluctuations, with blood pressure peaking during the dark cycle (12-24, 36-48 hrs) and declining during the light cycle (0-12, 24-36 hrs).

To further investigate these circadian differences, we compared blood pressure between the light and dark cycles across different genotypic groups (Figure 3.5B). The results confirmed a significant increase in MAP during the dark cycle compared to the light cycle within all groups ($P < 0.001$ or $P < 0.0001$). These findings indicate that germline or inducible MLK3 deletion both lead to a sustained increase in blood pressure while preserving normal circadian rhythmicity in blood pressure regulation.

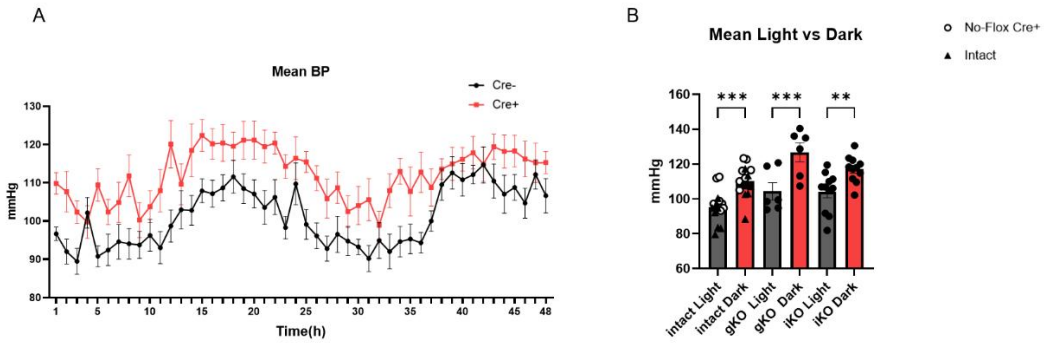


Figure 3.5 Normal light response of blood pressure in MLK3 inducible knockdown mice.

Blood pressure (BP) was recorded over 48 hours to assess the effects of genotype and circadian rhythms. (A) Time-course analysis of BP in MLK3 iKO (red) and MLK3 intact (black) mice. (B) BP comparison between light (gray) and dark (red) cycles across MLK3 intact, gKO, and iKO groups. n= 19 intact (n=8 No-Flox Cre+; n=11 MLK3fl/fl), n=7 gKO, n=10 iKO. Data were analyzed using two-way ANOVA with Šidák's multiple comparisons test. Error bars represent SEM. Statistical significance: ***, $P < 0.001$; **, $P < 0.01$; ****, $P < 0.0001$.

3.6 MLK3 inducible knockdown leads to reduced spleen mass.

To assess the potential impact of MLK3 deletion on organ development, organ weights were measured in MLK3 inducible knockout (iKO) mice and control (intact) mice. Measurements were normalized to tibia length

(TL) to account for variations in body size. Statistical analysis revealed no significant differences (ns) in body weight, tibia length, or other organ weights between the two groups (Figure 3.6 A, Table 1). The one exception was the spleen, which had significantly reduced mass in iKO mice ($P < 0.01$). These findings suggested that MLK3 deletion does not broadly affect overall organ development but may specifically influence spleen size (Figure3.6A), potentially indicating a role of MLK3 in immune or hematopoietic function.

To further investigate potential sex differences in body weight and organ size, we analyzed body weight and individual organ weight (normalized to tibia length) of MLK3-inducible knockout (iKO) mice and intact (control) mice, separated by sex. The results showed that in both genotypes, BW/TL and organ weights (except spleen) were significantly higher in males than in females ($P < 0.001$), indicating a sex difference in body weight and organ size (Figure 3.6 B). However, no significant differences in spleen size between males and females were observed across all genotypes (Figure 3.6 B). These findings suggest that MLK3 deletion does not alter sex-specific differences in body weight and organ size but does not affect spleen size in a sex-dependent manner.

Table 1 Organ weight data in fl/fl UBC-Cre- (iKO) and fl/fl UBC-Cre+ (Intact) mice

Parameter	Cre- (Intact), n=16	Cre+ (iKO), n=11	P value
Body weight (g)	28.11 ± 1.21	25.44 ± 1.74	ns
Tibia length (mm)	18.09 ± 0.14	18.03 ± 0.13	ns
LV (g)	0.09 ± 0.004	0.08 ± 0.004	ns
RV (g)	0.02 ± 0.001	0.02 ± 0.002	ns
LA (g)	0.004 ± 0.0002	0.003 ± 0.0004	ns
RA (g)	0.003 ± 0.0003	0.003 ± 0.0003	ns
Lung (g)	0.14 ± 0.01	0.13 ± 0.004	ns
Liver (g)	1.39 ± 0.07	1.24 ± 0.11	ns
Spleen (g)	0.09 ± 0.004	0.07 ± 0.004	0.02
Aorta (g)	0.004 ± 0.0004	0.004 ± 0.0003	ns
Kidney (g)	0.15 ± 0.01	0.13 ± 0.01	ns
BW/TL (g/mm)	1.54 ± 0.06	1.43 ± 0.07	ns
LV/TL (mg/mm)	5.08 ± 0.23	4.67 ± 0.20	ns
RV/TL (mg/mm)	1.04 ± 0.06	0.96 ± 0.07	ns
LA/TL mg/mm)	0.20 ± 0.01	0.17 ± 0.02	ns
RA/TL (mg/mm)	0.17 ± 0.01	0.16 ± 0.02	ns
Lung/TL (mg/mm)	7.69 ± 0.23	7.27 ± 0.19	ns
Liver/TL (mg/mm)	74.79 ± 3.69	69.47 ± 5.17	ns
Spleen/TL (mg/mm)	5.08 ± 0.23	4.04 ± 0.16	0.0025
Aorta/TL (mg/mm)	0.22 ± 0.02	0.22 ± 0.01	ns

Kidney/TL (mg/mm)	8.36 ± 0.35	7.62 ± 0.46	ns

Organ mass was measured on 12-14 weeks-old mice and normalized to tibia length (TL). Data were analyzed using two-tailed unpaired t-test and expressed as means ± SEM. LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium

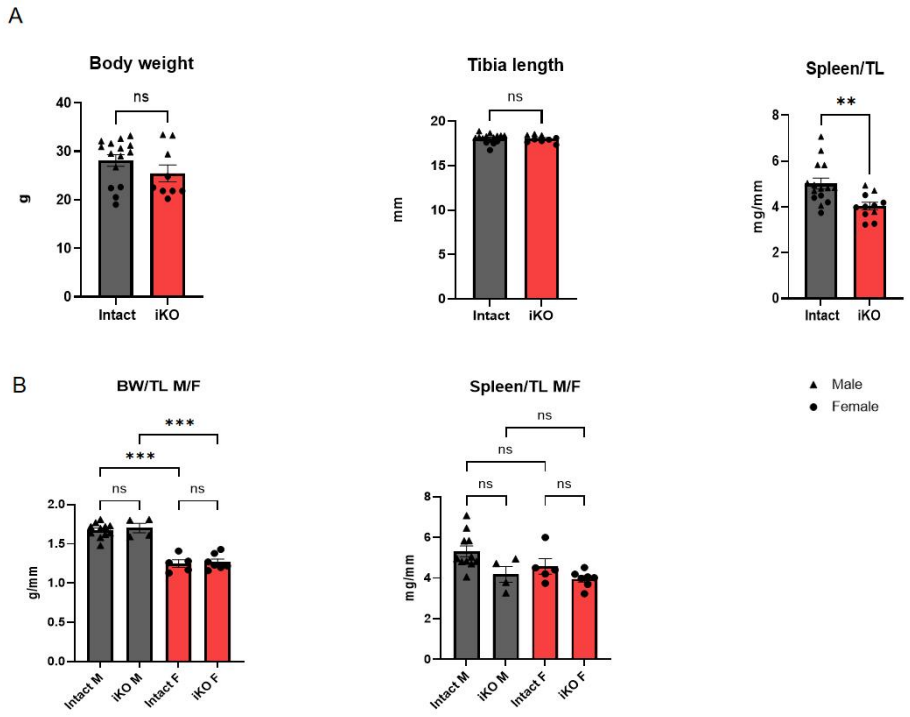


Figure 3.6 MLK3 inducible knockdown leads to reduced spleen mass. (A) Organ weights were measured in MLK3 inducible knockout (iKO) and control (intact) mice and normalized to tibia length (TL) (iKO: n=18; intact: n=11). Data are presented as means ± SEM and analyzed using an unpaired two-tailed t-test. ns, not significant; **, $P < 0.01$. BW/TL (g/mm) comparisons across iKO and intact groups for males (▲) and females (●).

Males exhibited significantly higher BW/TL than females in both genotypes ($P < 0.001$), but no significant genotype effect was observed. Spleen/TL (mg/mm) comparisons showed no significant differences across sexes or genotypes. (B) BW/TL (g/mm) and Spleen/TL (mg/mm) were compared between MLK3 inducible knockout (iKO) and control (intact) groups for males (▲) and females (●). BW/TL was significantly higher in males than females in both genotypes (***, $P < 0.001$), but no significant genotype effect was observed (ns, $P \geq 0.05$). Spleen/TL comparisons showed no significant differences across sexes or genotypes. Data are presented as mean ± SEM and analyzed using two-way ANOVA. ns, $P \geq 0.05$; *$P < 0.001$.

3.7 MLK3 inducible knockdown does not affect cardiac function or morphology

To determine the effect of MLK3 deletion on left ventricular (LV) function, we assessed systolic function, LV volume, and diastolic function in MLK3 inducible knockout (iKO) and control (intact) mice using echocardiography. Systolic function was evaluated by measuring ejection fraction (EF) and fractional shortening (FS), both of which showed no significant differences between iKO and control groups (Figure 3.7 A), indicating that MLK3 deletion does not impair LV contractility. LV mass

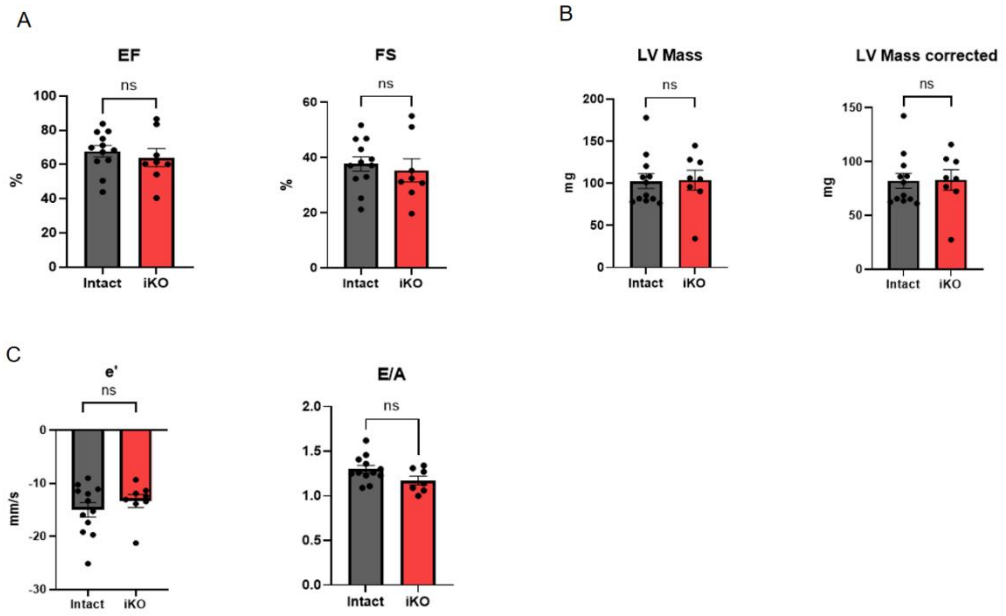
and LV volume were measured to assess structural changes (Figure 3.7 B), with no significant differences observed between genotypes, suggesting that MLK3 deletion does not alter cardiac morphology. Diastolic function was analyzed using early diastolic mitral annular velocity (e') and the E/A ratio (early-to-late ventricular filling velocity) (Figure 3.7 C), both of which showed no significant changes, indicating that diastolic function remains unaffected by MLK3 deletion. These results demonstrate that MLK3 deletion does not impact cardiac contractile function, LV volume, or diastolic function.

Table 2 Baseline LV structure and function in fl/fl UBC-Cre- (iKO) and fl/fl UBC-Cre+ (Intact) mice

Parameter	Cre- (Intact), n=12	Cre+ (iKO), n=8	P value
IVS;d (mm)	0.83 ± 0.03	0.77 ± 0.05	ns
LVID;d (mm)	3.55 ± 0.09	3.58 ± 0.20	ns
LVID;s (mm)	2.22 ± 0.12	2.34 ± 0.25	ns
LVPW;d (mm)	0.83 ± 0.08	0.86 ± 0.10	ns
EF (%)	67.74 ± 3.39	64.00 ± 5.31	ns
FS (%)	37.68 ± 2.57	35.36 ± 4.21	ns
LV Mass (mg)	102.80 ± 8.73	103.80 ± 11.80	ns
LV Mass corrected (mg)	82.25 ± 6.98	83.07 ± 9.44	ns
LV Vol;d (uL)	53.17 ± 3.61	55.43 ± 7.44	ns

LV Vol;s (uL)	17.57 ± 2.53	21.65 ± 5.86	ns
e' (mm/s)	-14.96 ± 1.37	-13.31 ± 1.24	ns
MV A (mm/s)	454.00 ± 39.50	453.70 ± 37.67	ns
MV E (mm/s)	577.60 ± 40.29	511.8 ± 43.57	ns
E/A	1.30 ± 0.04	1.17 ± 0.05	ns
E/e'	-40.06 ± 2.98	-41.01 ± 4.48	ns

Echocardiographic analysis was conducted in 12–14-week-old fl/fl UBC-Cre- (iKO) and fl/fl UBC-Cre+ (Intact) mice prior to organ harvest. Data were analyzed using two-tailed unpaired t-test and expressed as means ± SEM. IVS;d, Interventricular Septum in Diastole; LVID;d, Left Ventricular Internal Diameter in Diastole; LVID;s, Left Ventricular Internal Diameter in Systole; LVPW;d, Left Ventricular Posterior Wall in Diastole; EF, ejection fraction; FS, fractional shortening.



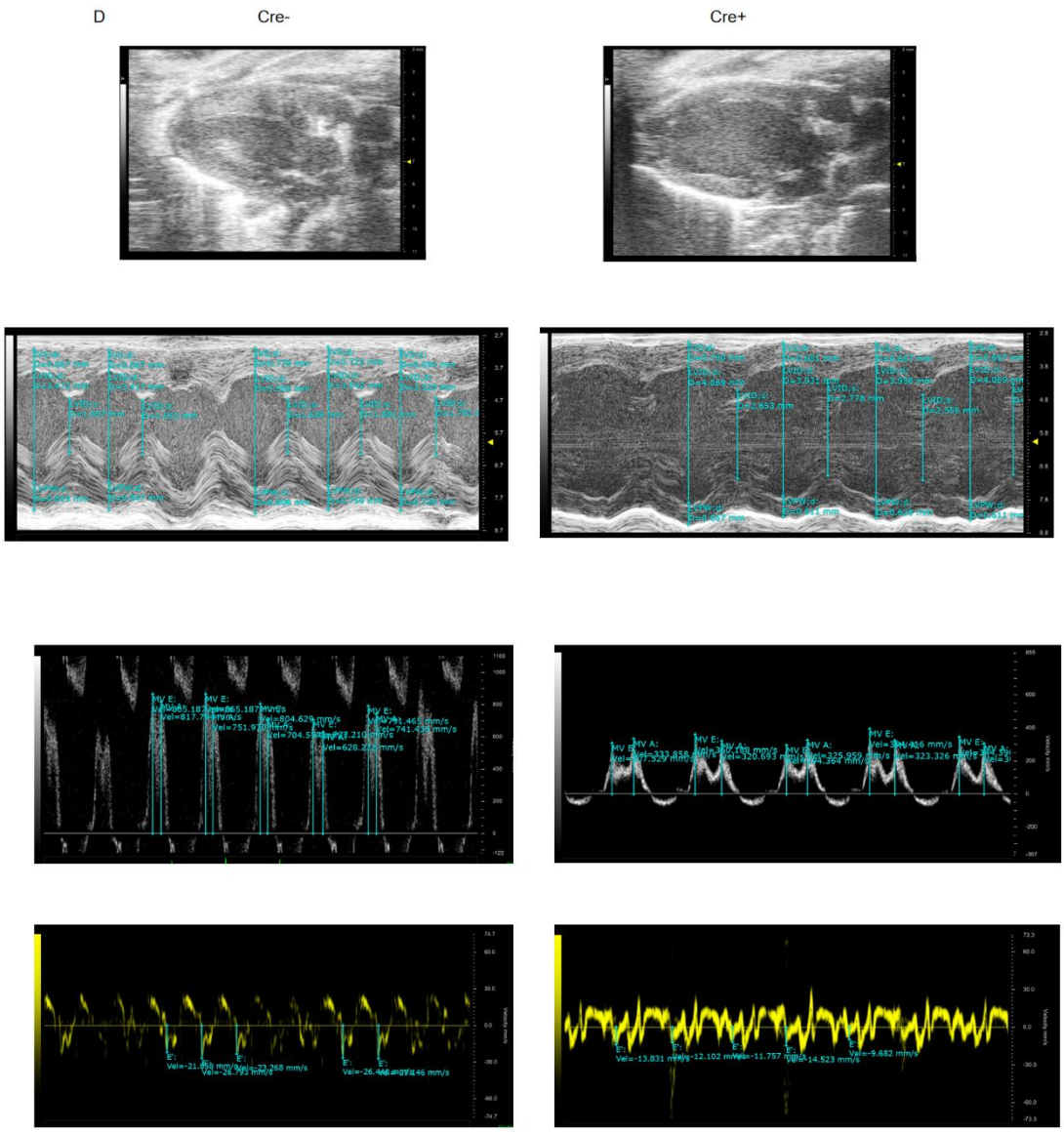


Figure 3.7 (A) Systolic functional parameters Ejection fraction (EF) and fractional shortening (FS) obtained by m-mode echocardiography in MLK3 inducible knockout (iKO) and intact control mice. (B) Left Ventricular Mass (LV) mass and corrected LV mass were also measured by echocardiography. (C) Diastolic parameters early diastolic mitral annular velocity (e') and the E/A ratio (early to late ventricular filling velocity). (D) Representative echocardiographic images from control

(Cre−) and MLK3 knockout (Cre+) mice, including B-mode, M-mode, and Doppler imaging. Intact: n=12, iKO: n=8 Data are presented as means ± SEM and analyzed using an unpaired two-tailed t-test. ns = not significant.

Chapter 4: Discussion

This presented study tested the hypothesis that MLK3 plays a critical role in blood pressure regulation and exerts blood pressure-independent effects on cardiac function. We found that whole-body inducible MLK3 knockout (iKO) leads to hypertension during both light and dark phases, emphasizing its essential role in maintaining blood pressure homeostasis. Despite this elevation in blood pressure, MLK3 deletion does not significantly alter cardiac structure or function, suggesting a distinct cardiovascular role beyond blood pressure regulation. Additionally, MLK3 deletion may have sex-specific effects, as male germline knockout (gKO) mice displayed a greater hypertensive response than females, while no significant sex differences were observed in iKO mice. Furthermore, MLK3 deletion led to reduced spleen size, suggesting a potential role in immune or hematopoietic function. Taken together, these findings support the hypothesis that MLK3 is a key regulator of blood pressure and cardiac function, with broader implications for vascular homeostasis and physiological regulation, providing insights into potential therapeutic strategies for hypertension and heart failure.

4.1 Tissue-Specific Expression of MLK3 and Its Potential Biological Significance

This study found that MLK3 is highly expressed in lung and spleen tissues, whereas its expression is lower in aortic tissues. Furthermore, MLK3 knockout resulted in a significant reduction of MLK3 protein levels in the lung and spleen, whereas no significant change was observed in the aorta of iKO mice, suggesting that MLK3 expression in these cardiovascular tissues may be regulated differently or compensated by other kinases.

The lung and spleen are highly active in immune and inflammatory responses, and as an upstream kinase in the MAPK signaling pathway, MLK3 may play a critical role in these processes. Previous studies have shown that MLK3 is involved in inflammatory signaling in macrophages and T cells, which could explain its high expression in the spleen and its potential role in immune regulation. Additionally, the prominent expression of MLK3 in lung tissue may be related to its function in epithelial cells or macrophages, particularly in responding to oxidative stress and inflammatory stimuli.

In contrast, the lack of significant MLK3 reduction in the aorta of iKO mice suggests that its role in vascular smooth muscle cells may be more limited or redundant with other signaling pathways. Since MLK3 is

primarily associated with cell proliferation, apoptosis, and inflammation, its relatively minor changes in this tissue could indicate that MLK3 is not a major regulator of basal vascular function under normal conditions. However, given its role in stress signaling pathways, further investigation is needed to determine whether MLK3 contributes to pathological remodeling or responses to cardiovascular stress in this tissue.

These findings suggest that MLK3 functions in a tissue-specific manner, playing a more prominent role in immune-related organs such as the lung and spleen, while its function in the cardiovascular system may be more subtle or compensated by alternative kinases. Future studies should explore MLK3's role in cardiac and vascular tissues under stress conditions, such as hypertension-induced remodeling or heart failure, to better understand its significance in cardiovascular health.

4.2 MLK3 in Blood Pressure Regulation: Mechanisms and Future Research Directions

Our findings demonstrate that MLK3 knockout results in significantly elevated systolic blood pressure (SBP) and mean arterial pressure (MAP), reinforcing the hypothesis that MLK3 plays a crucial role in blood pressure homeostasis. This aligns with previous studies indicating that MLK3 is involved in cardiovascular function by regulating vascular

smooth muscle contraction and cellular signaling pathways (Gao et al, 2023). Notably, the hypertensive phenotype observed in MLK3 knockout mice is consistent across both inducible and germline knockout models, suggesting that MLK3's role in blood pressure regulation is not developmentally restricted but persists in adulthood.

Mechanistically, MLK3 has been linked to the regulation of myosin light chain (MLC) phosphorylation, which plays a pivotal role in vascular smooth muscle contraction (Gao et al, 2023). Increased phosphorylation of MLC enhances vascular smooth muscle tone and elevates peripheral resistance, a key contributor to hypertension. In MLK3-deficient mice, enhanced MLC phosphorylation suggests that MLK3 may normally function to suppress excessive smooth muscle contraction, potentially through modulation of the RhoA/ROCK signaling pathway (Gao et al, 2023). Given that RhoA signaling is a major determinant of vascular tone, future studies should explore whether MLK3 interacts directly with this pathway and whether targeting MLK3 could be a viable strategy for modulating vascular resistance.

Additionally, our study indicates that the hypertensive effects of MLK3 deletion are more pronounced during the dark phase, suggesting a possible interaction between MLK3 and circadian regulation of blood pressure. This raises intriguing questions about whether MLK3 influences

sympathetic nervous system activity, renin-angiotensin system regulation, or endothelial function in a time-dependent manner (Gao et al, 2023). Future research could utilize tissue-specific MLK3 knockout models to distinguish its effects in vascular smooth muscle, endothelium, and neural control of blood pressure. Moreover, single-cell RNA sequencing of vascular tissues could provide insights into how MLK3 modulates gene expression networks in different cellular populations.

4.3 Potential cell types for MLK3's regulation of blood pressure

Our study demonstrates that postnatal MLK3 whole-body deletion leads to hypertension, indicating that MLK3 plays a crucial role in blood pressure homeostasis. However, the exact cell type(s) responsible for MLK3's regulation of blood pressure remain undetermined. Based on existing evidence, we hypothesize that MLK3 may exert its effects through vascular smooth muscle cells (SMCs), endothelial cells, or the brain, each of which plays a critical role in blood pressure regulation.

MLK3 is expressed in vascular smooth muscle cells (SMCs) (Gao et al, 2023), which play a fundamental role in blood pressure regulation by modulating vascular tone through vasoconstriction and vasodilation. The contractile state of SMCs is largely dependent on myosin light chain (MLC) phosphorylation (Gao et al, 2023), a process that enhances smooth

muscle tone and elevates peripheral resistance. MLK3 may influence SMC function by regulating MLC phosphorylation, possibly through modulation of the RhoA/ROCK signaling pathway. Loss of MLK3 has been shown to increase MLC phosphorylation, leading to excessive SMC contraction and elevated blood pressure (Gao et al, 2023). However, MLK3 deletion in SMCs alone does not lead to hypertension (unpublished data, Blanton Laboratory), suggesting that MLK3's effect on blood pressure may not be SMC-intrinsic or may require additional contextual cues. To test this directly, SMC-specific MLK3 knockout (SMC-KO) mice should be studied to determine whether MLK3 contributes to vascular contractility and blood pressure regulation through smooth muscle–specific mechanisms (Gao et al, 2023).

MLK3 is expressed in endothelial cells (ECs), where it may contribute to vascular homeostasis (Furuta et al, 2021). ECs are essential regulators of blood pressure, maintaining vascular tone through the release of vasodilators such as nitric oxide (NO) and prostacyclin, as well as vasoconstrictors like endothelin-1. MLK3 may influence EC function by modulating inflammatory and oxidative stress signaling pathways, which are known to affect NO production, endothelial barrier integrity, and mechanotransduction in response to blood flow. Impairment of MLK3 in ECs could lead to reduced NO bioavailability and increased vascular resistance, thereby contributing to elevated blood pressure. To

directly test the role of MLK3 in ECs, endothelial cell–specific MLK3 knockout (EC-KO) (Cadherin 5-Cre) mice should be used to examine changes in endothelium-dependent vasodilation, vascular tone, and overall blood pressure regulation (Furuta et al, 2021).

MLK3 is expressed in the brain, particularly in regions such as the hippocampus, where it plays a role in neuronal signaling and stress response pathways (Jin et al., 2010). The central nervous system, including the brainstem and hypothalamus, is essential for autonomic regulation of blood pressure via modulation of sympathetic outflow. MLK3 has been implicated in several neurodegenerative diseases, including Alzheimer's and Parkinson's disease, through its involvement in the MAPK signaling cascade and neuronal apoptosis (Jin et al., 2010) . Given this context, MLK3 may influence blood pressure centrally by affecting sympathetic tone or baroreceptor sensitivity. To test this hypothesis, neuron-specific MLK3 knockout mice targeting key autonomic control centers could be generated to evaluate whether loss of MLK3 disrupts central blood pressure homeostasis.

By investigating these three potential mechanisms through cell-type-specific knockout models, future studies can clarify whether MLK3 primarily regulates blood pressure through vascular smooth muscle, endothelial, or neural pathways. Understanding the precise role

of MLK3 in these systems will provide critical insights into its function in cardiovascular physiology and may offer new therapeutic targets for hypertension.

4.4 Clinical relevance

Our findings demonstrate that MLK3 is essential for maintaining blood pressure homeostasis and that its deletion leads to sustained hypertension, independent of structural cardiac changes. Unlike current antihypertensive agents that act broadly and often cause hypotension, MLK3 appears to modulate vascular tone through specific postnatal mechanisms involving RhoA/ROCK and myosin light chain signaling. This suggests that targeting MLK3 or its effectors may offer a novel therapeutic approach for hypertension with reduced risk of adverse hemodynamic effects. Given its role across multiple regulatory systems—including vascular smooth muscle, endothelium, and potentially the brain—MLK3 represents a promising candidate for selective modulation in resistant or multifactorial hypertension.

4.5 Limitations

The current study has several limitations. First, in the 6-month-old cohort, the MLK3 germline knockout (gKO) group included only two mice, limiting the statistical power and generalizability of findings related to long-term blood pressure effects. Although preliminary data suggest a sustained hypertensive phenotype in aged gKO mice, larger sample sizes will be necessary to robustly assess the persistence and potential progression of hypertension over time, as well as to examine sex-specific or circadian influences in the aging context. Second, although we employed a well-established tamoxifen-inducible Cre-loxP system to achieve postnatal MLK3 deletion, Western blot analysis indicated that only approximately 60–70% reduction in MLK3 protein was achieved in target tissues. This incomplete knockdown may reflect limitations in the five-day tamoxifen administration protocol. Future studies may benefit from extended tamoxifen regimens (e.g., two-week induction) to enhance recombination efficiency and achieve more complete gene excision, thereby allowing for a clearer interpretation of MLK3's physiological role. Third, as with most murine studies, translational relevance to human biology remains a challenge. Although MLK3 polymorphisms have been associated with blood pressure variation in human populations, the functional role of MLK3 in human cardiovascular regulation cannot be

directly assessed in vivo. Further research utilizing human-derived vascular cells or organoid systems, as well as population-level genetic and pharmacogenomic analyses, will be essential to determine whether MLK3-targeted therapies hold promise for clinical application.

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